University of Granada

Ph.D. Thesis

Evaluation of a polyphenol-rich orange juice on metabolic syndrome and cardiovascular risk biomarkers in humans with overweight or obesity.

Evaluación de una bebida de naranja rica en polifenoles sobre marcadores de síndrome metabólico y riesgo cardiovascular en humanos con obesidad o sobrepeso

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III. Rangel-Huerta OD, Aguilera CM, Pérez-de-la-Cruz A, Gil A, Mesa MD. Impact of a flavanone-rich orange juice consumption on oxidative stress and inflammation: a metabolomics approach. (manuscript pending to be submitted)

SUMMARY

Background

Overweight and obesity are such a world pandemic. The accumulation of visceral adiposity present in obesity has been strongly associated with insulin resistance (IR), hypertension and dyslipidaemia, increasing rates of morbidity and mortality. Additionally, a decrease in protective factors, such as adiponectin, and the dysregulation of inflammatory molecules could lead to a chronic low-grade inflammatory status and, later on, to metabolic syndrome (MS) (Cañete, Gil-Campos, Aguilera, & Gil, 2007; Lankinen *et al.*, 2010)

Obesity may induce systemic oxidative stress, which is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism. ROS are a variety of structures, free radicals and non-radicals, such as superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), generated by normal cellular metabolism and by exogenous agents that may serve as cell signalling or bactericidal agents. An excess of ROS formation and/or a deficient antioxidant capacity causes extensive damage in cellular macromolecules such as polyunsaturated lipids, proteins and DNA (Castilla-Cortázar 2012). In fact, the production of ROS in adipose tissue can produce an increase in inflammation, dysregulation of adipocytokines and the migration of oxidative stress to remote tissues. Through these mechanisms, ROS contribute to the progress of IR, diabetes or atherosclerosis and developing the MS (Fernández-Sánchez *et al.* 2011; Rupérez, Gil, and Aguilera 2014).

When obesity is constant across the time, there is an unbalance in ROS production; consequently, antioxidant sources can be depleted. The enzymatic antioxidant defence system (E-ADS) includes, but is not limited to, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). On the other hand, there is a non-enzymatic antioxidant defence system (NEADS), which mostly help regenerate glutathione disulphide (GSSG) back into glutathione (GSH). Antioxidant vitamins such as A, C, E and alpha-lipoic acid are among these mechanisms.

DNA, lipids, proteins and carbohydrates are examples of molecules that can be modified by excessive ROS in vivo and often those are used as biomarkers. The F2-isoprostanes (8-iso-PGF2α) in urine, and malondialdehyde (MDA) and oxidised low-density lipoprotein (oxLDL)

in plasma are examples of well-established lipid peroxidation biomarkers. In addition to the cytotoxic effect of lipid peroxidation, oxidative DNA damage occurs immediately and constantly. In recent years, 8-hydroxy-2'deoxiguanosine (8-OHdG) emerged as a reliable marker of oxidative stress in DNA induced by ROS, as it is a major product formed by hydroxyl radical attack to the DNA base guanine.

In addition, inflammation has been recognized as a major risk factor for diverse human diseases. Augmented visceral adiposity as in obesity is associated with a higher production of proinflammatory adipocytokines such as leptin, tumour necrosis alpha (TNF-α) and interleukins (IL) IL-1, IL-6 and IL-8 (Guzik, Mangalat, and Korbut 2006). Deregulated production of these molecules participates in the pathogenesis of MS (Medzhitov 2008).

Flavonoids are secondary metabolites that may be found in fruits, vegetables and beverages derived from plants. These molecules are powerful in vitro antioxidants with pharmacological properties such as antithrombotic and anti-inflammatory (Landberg *et al.* 2011; Kim *et al.* 2011). Citrus fruits contain approximately 95% of the total flavonoids encompassed mainly in flavanones, flavones and flavonols subclasses (Bahorun *et al.* 2012). Particularly, orange juice (OJ) contains the flavanones glycosides: hesperidin (200-600 mg/L) and narirutin (15-85 mg/L). Furthermore, the consumption of citrus juices enriched in flavanones is associated with decrease of incidence in coronary heart disease, blood cells DNA oxidative damage, Apo-B concentration (principal component of LDL-cholesterol), LDL oxidability and with the improvement of plasma concentration of inflammation and vascular function biomarkers (Morand *et al.* 2011; S. Sharma *et al.* 2012; A. K. Sharma *et al.* 2011; Mulvihill *et al.* 2009; Wilcox *et al.* 2001; Borradaile, Carroll, and Kurowska 1999; Rizza *et al.* 2011; Miwa *et al.* 2005; Jung *et al.* 2003; Buscemi *et al.* 2012; Devaraj *et al.* 2011; Gardana *et al.* 2007; Giordano *et al.* 2011). Recently, as part of this doctorate work, our research group developed a systematic review of bioactive compounds in the cardiovascular disease already accepted in Nutrients. In this review, we covered several polyphenols groups and we included 59 papers studying flavonoids. However, using our search equations, we did not find any publication approaching the effect of flavanones. We think that is necessary to study in deep the effect of flavanones in overweight and obesity risk comorbidities such as inflammatory and oxidative stress parameters as well as in the metabolic syndrome components.

Metabolomics is a scientific analysis that uses a systematic pipeline of the unique chemical fingerprints present in a target organism using innovative analytical technologies. Many studies using metabolomics have observed the effects of certain dietary patterns or the inclusion of particular dietary products in health and disease; this is known as nutrimetabolomics (Guertin *et al.* 2014; Suhre 2014; Schäfer *et al.* 2014). The discovery of dietary exposure biomarkers and altered metabolites may serve as diagnostic tool and enable preventive action. The possibility to discover new biomarkers of polyphenol consumption represents an interesting approach for unravelling their protective effects in human health. Those protective mechanisms involves diverse regulation pathways at the molecular/cellular level as well as direct antioxidant properties. Thus, metabolomics help to identify the complex and subtle influences on whole body metabolism and physiology.

Rationale of the study

The research group CTS-461 "Biochemistry of Nutrition. Therapeutic Implications" develop its research in lines including childhood and adult obesity through different approaches, which include the study of novel obesity, inflammatory and oxidative stress biomarkers.

The first approach was to compare the effect of supplementation with two different OJs, enriched with different doses of polyphenols, on MS and cardiovascular disease (CVD) risk factors, E-ADS and NEADS systems and on inflammatory and oxidative stress biomarkers in overweight and obese adults. Additionally, the use of the state-of-the-art technology metabolomics was included, trying to identify new biomarkers related to the OJ consumption.

Study design

A randomised, crossover, double blind (subjects and investigators), 12-wk dietary intervention trial was conducted with OJs containing the following two different polyphenol levels: i) 0.6 mg/ml, OJ with the normal polyphenol content (NPJ) and ii) 1.5 mg/ml, OJ with the high polyphenol content (HPJ). A 7-wk washout period was used between the 12-wk consumption of each juice. The subjects were randomly assigned to each of the two groups, which were paired according to sex and age, using a random number generator program. The first group (n = 54) received 2 daily doses (250 ml each) of the HPJ for 12-wk (corresponding to a daily dose of 582.5 mg of hesperidin, 125 mg of narirutin and 34 mg of didymin). After a 7-

wk washout period, the subjects received the NPJ daily (corresponding to 237 mg of hesperidin, 45 mg of narirutin and 17 mg of didymin). The second group (n = 46) received the NPJ for 12-wk followed by a 7-wk washout period, after which they received the HPJ for 12-wks.

Methodology

Blood samples were collected in the fasting state. After centrifugation, serum and plasma specimens were frozen at −80°C. The erythrocyte pellet was washed and frozen at −80°C to ensure lysis. First-morning urine from subjects was collected and aliquots were stored at −80 ºC until subsequent analyses.

Blood pressure (systolic and diastolic blood pressure -SBP and DBP, respectively) and anthropometric measurements were performed by standardised methods, and blood samples were drawn after overnight fasting. A general serum biochemical analysis was run at the participating hospitals. Plasma adipokines and biomarkers of inflammation and endothelial damage (leptin, IL-1β, IL-6, IL-8, TNF-α and t-PAI1) were measured by Luminex 200 equipment using the xMap technology. In addition, urinary 8-OHdG, 8-iso-PGF2α and oxLDL were measured by ELISA. Plasma LPO was analysed using a colorimetric kit and MDA using a TBARS assay kit. Plasma retinol, α-tocopherol and ß-carotene levels were measured by high-pressure liquid chromatography (HPLC). The E-ADS was evaluated by catalase, SOD, GR and GPX activities. The determination of urine hesperedin, narirutin and their metabolites was performed using a UHPLC system.

A linear mixed-effects model (LMM), with the intercept as random effect and a covariance structure for repeated measures by time and OJ, was used to determine the differences between the interventions. Correlations between the concentrations of the main flavanones and variables were estimated by the Pearson's correlation coefficient when the assumptions of normality were met and by the Spearman's correlation coefficient when the assumptions of normality were not met. $P < 0.05$ was considered as significant.

A subsample of 30 subjects, aged 22-63 y were selected. Global biochemical profiles were determined by Metabolon Inc. (NC, USA), in human serum, representing gender and age matched treatment groups collected at initial baseline and final time points. The analysis was developed using UPLC-MS/MS with positive and negative ion mode, electrospray ionization, a LC polar platform, and GC-MS. Raw data was extracted, peak-identified and QC processed

using specific Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Different statistical methods were used for exploring metabolomics data and identify patterns, such as principal component analysis (PCA) and Random Forest analysis (RF). For univariate analysis, a t-test for independent samples was carried out identify possible differences at baseline between groups. For the analysis of variance (ANOVA), three types of effect were determined, time (basal vs final), treatment (NPJ vs HPJ) and time x treatment interaction. The false discovery rate (q) was calculated for avoid the discovery of false positives and a cut-off < 0.1 was set up.

Results

Here we summarize the more relevant results of the study. The intake of either NPJ or HPJ led to a decrease in urinary 8-OHdG, 8-iso-prostaglandin as well as the erythrocyte catalase and GR activities. A decrease was also observed in BMI, waist circumference and leptin (all $P < 0.05$) after the 12-wk interventions with both OJs. Only the NPJ intervention decreased systolic and diastolic blood pressure. Finally, the HPJ group had increased erythrocyte SOD activity.

Using the metabolomics approach, six hundred fifty-one metabolites were identified, 33 corresponding to the GC-MS platform, 321 corresponding to the LC/MS positive mode, 221 corresponding to the LC/MS negative mode and 76 corresponding to the LC/MS polar mode. There were no significant differences between interventions nor intervention x time interaction. However, 79 metabolites shown a significant time effect ($p \leq 0.05$; $q \leq 0.1$). After applying the PCA and the unsupervised hierarchical analysis, clustering failed to differentiate between LPJ and HPJ basal subject. However, when utilizing RF analysis, a unique differential biochemical signature was observed between HPJ baseline and post-HPJ samples. In this case, the predictive accuracy was 97%. Interestingly, three of the top five metabolites found in this metabolic signature are those related to OJ consumption, i.e. methyl glucopyranoside (alpha-beta), stachydrine and betonicine.

The major lipoxygenation products derived from linoleic acid, 9-hydroxy-10,12-octadecadienoic acid (9-HODE) plus 13-hydroxy-9,11-octadecadienoic acid (13HODE) were significantly diminished only after the 12-wk intervention with the HPJ (FC: 0.50; $q = 0.0421$). These metabolites were correlated with 9,10-dihydroxy-octadecenoic acids (9,10-DiHOME) and

12,13-DiHOME (rho =0.401; p = 0.028 and rho = 0.449; p = 0.013, respectively) and inversely correlated with betonicine (rho = -0.399; p = 0.029) and naringin (rho = -0.428; p = 0.018).

Conclusion

In conclusion, our results demonstrate that the consumption of either NPJ or HPJ protected against DNA damage and lipid peroxidation, modified several antioxidant enzymes and improved body weight in overweight or obese non-smoking adults. Only blood pressure and SOD activity were influenced differently by the different flavanone supplementations.

The use of metabolomics could give a deeper insight in nutritional interventions, as we verified it is possible to determine biomarkers of OJ consumption and to assess the validity of the dietary intervention and also go further and determine the effects in health and pathology that are not possible to find with traditional biomarkers and help us to provide a better dietary advice. It is necessary to expand the strategy to a greater population and validate the results obtained in the present thesis. The elucidation of the specific role of each flavanone and their mechanisms of action will require further studies.

Study strength and limitations

The following limitations should be taken into account. We did not have a control (placebo) group that help us to compare the results of the two different doses of polyphenols to free living subjects. In addition, BMI decrease could be a confounder factor for the outcomes we were looking, due to the relationship between obesity and inflammation. A determinant in crossover trials is the carryover effect that could be related to fatigue or short-term for washout and is reflected as a different response after the two periods of intervention. In our statistical analysis, we adjusted for the BMI and calculated the carryover effect in order to manage adequately these limitations. In some cases, we found a significant P-interaction (treatment per time interaction) that showed that there was still a carryover effect after the 7-wk washout period that avoids seeing the real effects in some outcomes.

In addition, the use of a small sample size in the metabolomics analysis may limit the effects shown in the study; a greater size is required to confirm the results obtained in this work.

RESUMEN

Antecedentes

El sobrepeso y la obesidad son una pandemia mundial. La acumulación de la adiposidad visceral presente en la obesidad ha sido fuertemente asociada con la resistencia a la insulina (RI), la hipertensión, la dislipidemia y el aumento de las tasas de morbilidad y mortalidad. Además, una disminución en factores protectores, tales como la adiponectina, y la desregulación de moléculas inflamatorias podrían conducir a un estado inflamatorio crónico de bajo grado y, más tarde, al síndrome metabólico (SM) (Cañete *et al.* 2007; Lankinen *et al.* 2010).

La obesidad puede inducir estrés oxidativo sistémico, que se define como un desequilibrio entre las especies reactivas de oxígeno (ROS) y los sistemas de producción antioxidante en el organismo. Los ROS son una variedad de estructuras, que incluyen radicales libres peróxidos, tales como el anión superóxido ($O_2^{\bullet -}$), el peróxido de hidrógeno (H_2O_2), y los radicales hidroxilo (OH^-), generados por el metabolismo celular en estado normal y por agentes exógenos que pueden servir como señalización celular o agentes bactericidas. Un exceso en la formación de ROS y/o una capacidad antioxidante deficiente provoca grandes daños en las macromoléculas celulares, tales como los lípidos poliinsaturados, las proteínas y el ácido desoxirribonucleico (ADN) (Castilla-Cortázar 2012). De hecho, la producción de ROS en el tejido adiposo puede producir un aumento en la inflamación, la desregulación de adipocitoquinas y la migración del estrés oxidativo a los tejidos remotos. A través de estos mecanismos, las ROS contribuyen al progreso de la IR, la diabetes o la aterosclerosis y en el desarrollo del SM (Fernández-Sánchez *et al.* 2011; Rupérez, Gil, y Aguilera 2014).

Cuando la obesidad es constante a través del tiempo, existe un desequilibrio en la producción de ROS; en consecuencia, las fuentes de antioxidantes se pueden agotar. El sistema de defensa antioxidante enzimático (E-ADS) incluye, pero no se limita a, la superóxido dismutasa (SOD), la catalasa y la glutatión peroxidasa (GPX). Por otro lado, existe un sistema de defensa antioxidante no enzimático (NEADS), que en su mayoría ayudar a regenerar el disulfuro de glutatión (GSSG) de nuevo en glutatión (GSH). Las vitaminas antioxidantes como la A, C, E y el ácido alfa lipoico son algunos de estos mecanismos.

El ADN, los lípidos, las proteínas y los hidratos de carbono son ejemplos de moléculas que pueden ser modificadas por el exceso de ROS in vivo y con frecuencia son utilizados como biomarcadores. Los F2-isoprostanos (8-iso-PGF2α) en la orina, el malonaldehído (MDA) y las lipoproteínas de baja densidad oxidadas (oxLDL) en el plasma son ejemplos de biomarcadores de la peroxidación lipídica bastante fiables. Además del efecto citotóxico de la peroxidación lipídica y el daño oxidativo del ADN se produce inmediatamente y constantemente. En los últimos años, la 8-hidroxi-2'deoxiguanosina (8-OHdG) surgió como un marcador fiable de estrés oxidativo en el ADN inducido por las ROS, ya que es un producto principal formado por el ataque de los radicales hidroxilo a la base guanina del ADN.

Así mismo, la inflamación ha sido reconocida como un factor de riesgo importante para diversas enfermedades humanas. El aumento de la adiposidad visceral como se presenta en la obesidad, se asocia con una mayor producción de adipocitoquinas proinflamatorias tales como la leptina, y el factor de necrosis tumoral alfa (TNF-α) y las interleucinas (IL) IL-1, IL-6 y IL-8 (Guzik, Mangalat, y Korbut 2006). La producción descontrolada de estas moléculas forma parte de la patogénesis de la MS (Medzhitov 2008).

Por otro lado, los flavonoides son metabolitos secundarios que se pueden encontrar en frutas, verduras y bebidas derivadas de plantas. Estas moléculas poseen propiedades antioxidantes in vitro con y además propiedades farmacológicas tales como antitrombóticos y anti-inflamatorios (Landberg *et al.* 2011; Kim *et al.* 2011). Las frutas cítricas contienen aproximadamente 95% de los flavonoides totales, compuestas principalmente por las subclases: flavanonas, flavonas y flavonoles (Bahorun *et al.* 2012). Particularmente, el zumo de naranja contiene los glucósidos de las flavanonas: hesperidina (200-600 mg/l) y narirutina (15-85 mg/l). Además, el consumo de zumos de cítricos enriquecido en flavanonas se asocia con la disminución de la incidencia en la enfermedad coronaria del corazón, el daño oxidativo del ADN, la concentración de Apo-B (componente principal de LDL-colesterol), oxidabilidad LDL y la mejora de la concentración plasmática de biomarcadores de inflamación y de función vascular (Morand *et al.* 2011; Sharma *et al.* 2012; Sharma *et al.* 2011; Mulvihill *et al.* 2009; Wilcox *et al.* 2001; Borradaile, Carroll y Kurowska 1999; Riza *et al.* 2011; Mida *et al.* 2005; Jung *et al.* 2003; Buscemi *et al.* 2012; Devaraj *et al.* 2011; Gardana *et al.* 2007; Giordano *et al.* 2011). Recientemente, como parte de este trabajo de doctorado, nuestro grupo de investigación desarrolló una revisión sistemática relacionada con compuestos bioactivos en la enfermedad cardiovascular y que ha sido aceptada en la revista

Nutrients. En esta revisión, cubrimos varios grupos de polifenoles y se incluyeron 59 trabajos que estudiaban los flavonoides. Sin embargo, con las ecuaciones de búsqueda empleadas no fue posible encontrar publicaciones relacionadas con las flavanonas. Por tanto, creemos que es necesario estudiar en profundidad el efecto de flavanonas en las comorbilidades de riesgo del sobrepeso y la obesidad, tales como parámetros inflamatorios y de estrés oxidativos, así como en los componentes del SM.

La metabolómica es un análisis científico que utiliza una estrategia sistemática para el análisis de las huellas bioquímicas presentes en un organismo diana usando tecnologías analíticas innovadoras. Muchos estudios que han utilizado la metabolómica han observado los efectos de ciertos patrones dietéticos o la inclusión de determinados productos de la dieta en la salud y la enfermedad, y a esto se le conoce como nutrimetabolómica (Guertin *et al.* 2014; Suhre 2014; Schäfer *et al.* 2014). El descubrimiento de biomarcadores de exposición dietética y la modificación de metabolitos puede servir como herramienta de diagnóstico y por tanto, permitir llevar a cabo acciones preventivas. La posibilidad de descubrir nuevos biomarcadores de consumo de polifenoles representa un enfoque interesante para desentrañar sus efectos protectores en la salud humana. Esos mecanismos de protección implican diversas vías de regulación a nivel molecular/celular, así como propiedades antioxidantes. Es por eso que la metabolómica puede ayudar a identificar las complejas y sutiles influencias en el metabolismo de todo el cuerpo y la fisiología asociadas con el consumo del zumo de naranja.

Justificación del estudio

El grupo de investigación CTS-461 "Bioquímica de la Nutrición. Implicaciones terapéuticas "desarrolla su investigación en diversas líneas incluyendo: la obesidad infantil y adulta a través de diferentes enfoques que incluyen el estudio de la obesidad a través de novedosos biomarcadores inflamatorios y de estrés oxidativo.

El primer objetivo fue comparar el efecto de la suplementación con dos diferentes zumos de naranja enriquecidos con diferentes dosis de polifenoles, en el SM y la enfermedad cardiovascular (ECV) los factores de riesgo, y en los sistemas NEADS y E-ADS, así como en biomarcadores de estrés oxidativo y de inflamación en adultos con sobrepeso y obesidad. Además, se incluyó el uso de la metabolómica con la finalidad de identificar nuevos biomarcadores relacionados con el consumo de zumo de naranja.

Objetivos del estudio

El principal objetivo fue comparar el efecto de la suplementación con dos zumos de naranja enriquecidos con diferentes dosis de polifenoles: uno con contenido normal (NPJ) (0,6 mg/m L) y otro con un alto contenido (HPJ) (1,5 mg/ml) en factores de riesgo ECV, MS, en el NEADS y E-ADS, así como en biomarcadores de estrés oxidativo en voluntarios con sobrepeso y obesidad.

Diseño del estudio

Se trata de un estudio con diseño cruzado, aleatorizado, a doble ciego (sujetos e investigadores), con una intervención dietética de 12 semanas y un periodo de lavado intermedio de 7 semanas. Los sujetos fueron asignados aleatoriamente a cada uno de los dos grupos, y fueron emparejados por sexo y edad, mediante un generador de números aleatorios. Los sujetos se dividieron en dos grupos que consumieron dos zumos de naranja con dos concentraciones de polifenoles distintas: i) 0,6 mg/ml, zumo de naranja con un contenido normal de polifenoles (NPJ) y ii) 1,5 mg/ml, un zumo de naranja con alto contenido en polifenoles (HPJ). El primer grupo (n = 54) recibió 2 dosis diarias (250 ml cada una) del HPJ durante 12 semanas (correspondiente a una dosis diaria de 582,5 mg de hesperidina, 125 mg de narirutina y 34 mg de didimina). Después del período de lavado de 7 semanas, los sujetos recibieron el NPJ diario (correspondientes a 237 mg de hesperidina, 45 mg de narirutina y 17 mg de didimina). El segundo grupo (n = 46) recibió el NPJ durante 12 semanas, a continuación de un período de lavado de 7 semanas, después del cual recibieron el HPJ por 12 semanas.

Metodología

Las muestras de sangre fueron recogidas en ayunas. Después de centrifugar, las muestras de suero se procesaron en el Hospital Virgen de las Nieves para el análisis de parámetros bioquímicos y el plasma se congeló a -80 ° C. El pellet de eritrocitos se lavó y se congeló a -80 ° C para asegurar la lisis. Se tomó la primera orina de la mañana y las alícuotas se almacenaron a -80 ° C hasta su posterior análisis.

La presión arterial (sistólica (PAS) y diastólica (PAD), respectivamente) y las mediciones antropométricas se realizaron por métodos estandarizados. Se realizó una medición de adipoquinas y biomarcadores de inflamación y daño endotelial (leptina, IL-1β, IL-6, IL-8, TNF-

α y t-PAI1) en plasma. Las mediciones se realizaron utilizando el equipo Luminex 200 a través de la tecnología xMap. Además, los marcadores 8-OHdG, 8-iso-PGF2α y oxLDL se midieron por ELISA en orina. La peroxidación lipídica en plasma se analizó utilizando un kit colorimétrico y el MDA usando un kit de ensayo de TBARS. El retinol, tocoferol α y los niveles de ß-caroteno se midieron por cromatografía líquida de alta presión (HPLC) en plasma. El sistema de defensa antioxidante fue evaluado mediante las actividades de la catalasa, SOD, GR y GPX en eritrocitos. La determinación de hesperedina, narirutina y sus metabolitos se realizó utilizando un sistema de cromatografía de líquidos de ultra alta resolución (UHPLC) en muestras de orina.

Se utilizó un modelo lineal de efectos mixtos (LMM) para determinar las diferencias entre las intervenciones, con el intercepto como efecto aleatorio y una estructura de covarianza para medidas repetidas por tiempo y la intervención, Las correlaciones entre las concentraciones de los principales flavanonas y variables fueron estimadas por el coeficiente de correlación de Pearson cuando se cumplían los supuestos de normalidad y por el coeficiente de correlación de Spearman cuando no se cumplían los supuestos de normalidad. $P < 0,05$ fue considerado significativo.

Además, se seleccionó una sub-muestra de 30 sujetos, con una edad de entre 22-63 años. Los análisis fueron elaborados por Metabolon Inc. (NC, EE.UU.), en suero humano, pareados por edad y sexo. Se tomaron muestras del tiempo basal y final correspondientes al primer brazo del estudio (diseño paralelo).

El análisis fue desarrollado utilizando cromatografía liquida de ultra precisión/espectrometría de masas (UPLC-MS/MS) con el modo de ion positivo y negativo, mediante ionización por electrospray, en una plataforma polar de cromatografía liquida (LC), y cromatografía de gases/espectrometría de masas (GC-MS). Los datos en bruto se extrajeron, los picos fueron identificados y se utilizaron controles de calidad. El procesado de datos se realizó utilizando hardware y el software propietario de Metabolon. Los compuestos fueron identificados por comparación con estándares de la base de datos de Metabolon. Diferentes métodos estadísticos se utilizaron para explorar los datos de metabolómica y en búsqueda de identificar patrones, entre ellos el análisis de componentes principales (PCA) y el análisis de "selvas aleatorias" (RF). Para el análisis univariante, se llevó a cabo un t-test para muestras independientes para identificar la posible diferencia a tiempo basal entre grupos. Para el análisis de la varianza (ANOVA), tres tipos de efectos se determinaron, el tiempo (basal vs final), tratamiento (NPJ vs HPJ) y la

interacción tratamiento x tiempo. Se calculó la tasa de falsos positivos (q) y se estableció un punto de corte < 0.1.

Resultados

Se resumen los resultados más relevantes del estudio. La ingesta de cualquiera, el NPJ o el HPJ, condujo a una disminución de 8-OHdG en orina, de 8-Iso-PGF2α, así como las actividades catalasa y GR en eritrocito. También se observó una disminución en el IMC, la circunferencia de la cintura y la leptina (todos $p < 0,05$) tras 12 semanas con ambos zumos de naranja. Por otro lado, sólo la intervención con el NPJ disminuyó presión arterial sistólica y diastólica. Finalmente, el grupo HPJ mostró un aumento en la actividad de la SOD en eritrocitos.

Utilizando el análisis metabolómico, se identificaron seiscientos cincuenta y un metabolitos, 33 correspondiente a la plataforma de GC-MS, 321 correspondiente al modo positivo LC/MS, 221 que corresponde a la LC/MS modo negativo y 76 que corresponde a la LC/MS modo polar. No hubo diferencias significativas entre las intervenciones ni interacción tiempo x intervención. Sin embargo, 79 metabolitos mostraron un efecto tiempo significativo ($p \leq 0,05$; $q \leq 0,1$). Después de utilizar el modelo PCA, la agrupación no logró identificar patrones metabólicos distintos entre sujetos de los grupos LPJ y HPJ. Sin embargo, cuando se utiliza el análisis de RF, se observó una firma bioquímica diferencial única entre las muestras basales y finales del grupo HPJ. En este caso, la precisión predictiva fue del 97%. Es de interés mencionar que tres de los cinco principales metabolitos encontrados en esta firma metabólica se encuentran relacionados con el consumo de zumo de naranja, siendo estos el metil glucopiranósido (alfa-beta), la estaquidrina y la betonicina.

Los principales productos lipoxigenados derivados de ácido linoleico, 9-hidroxi-10,12-octadecadienoico ácido (9-HODE), además del 13-hidroxi-9,11-octadecadienoico ácido (13-HODE) disminuyeron significativamente sólo tras 12 semanas de intervención con el HPJ (FC: 0,50; $q = 0,0421$). Además, estos metabolitos se correlacionaron con los ácidos-9,10-dihidroxi-octadecenoico (9,10 DiHOME) y 12,13-DiHOME (rho = 0,401; $p = 0,028$ y rho = 0,449; $p = 0,013$, respectivamente) y mostraron una correlación inversa con la betonicina (rho = -0,399; $p = 0,029$) y la naringina (rho = -0,428; $p = 0,018$).

Conclusión

En conclusión, nuestros resultados demuestran que el consumo de cualquiera de los dos zumos, protege contra el daño del ADN y la peroxidación lipídica, también se modificaron varias enzimas antioxidantes y se mejoró el peso corporal en adultos no fumadores con sobrepeso u obesidad. Mientras que la presión arterial y la actividad de la SOD se modificaron únicamente tras la ingesta del HPJ.

El uso de la metabolómica puede dar una visión más profunda en las intervenciones nutricionales, como hemos comprobado, es posible determinar biomarcadores de consumo de zumo de naranja y de evaluar la validez de la intervención dietética. También es posible ir más allá y determinar los efectos en la salud y en la patología que no son posibles de descubrir con los biomarcadores tradicionales. Por tanto nos puede ayudar a proporcionar un mejor asesoramiento dietético. Es necesario aplicar lo expuesto aquí en una población mayor y validar los resultados obtenidos en la presente tesis. La elucidación de la función específica de cada flavanona y sus mecanismos de acción requiere de más estudios.

Fortalezas y limitaciones del estudio.

Las siguientes limitaciones deben tenerse en cuenta. Nuestro diseño carecía de un grupo control (placebo) que podría servir para evaluar la comparación de las dos dosis de polifenoles presentadas frente a sujetos exentos de tratamiento.

Además, la disminución del IMC podría ser una variable confusora con influencia en nuestros objetivos principales, debido a la relación que tiene esta variable con la obesidad y la inflamación. Asimismo, un factor determinante en los ensayos cruzados es el efecto de arrastre que podría estar relacionado con la fatiga o el corto plazo del periodo de lavado, esto se refleja en una respuesta diferente entre los dos periodos de intervención.

En nuestro análisis estadístico, realizamos un ajuste por el índice de masa corporal y se calculó el efecto de arrastre con el fin de gestionar adecuadamente estas limitaciones. En algunos casos, encontramos una interacción significativa (interacción tiempo por tratamiento) que demostró que existía un efecto de arrastre aún después del período de lavado de 7 semanas. Esto podría ser un factor de confusión que evitaría ver los efectos reales en algunos resultados.

Por último, dado el pequeño tamaño de muestra empleado en el análisis de metabolómica, los efectos se podrían ver limitados, por tanto, se requiere un mayor tamaño de muestra para confirmar los resultados obtenidos en el presente trabajo.

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BACKGROUND

BACKGROUND

Obesity and metabolic syndrome

Overweight and obesity are considered the world pandemic for the 21st Century. Worldwide obesity has nearly doubled since 1980. In 2012, the World Health Organisation estimated that overweight in adults, 20 and older, exceeded 1.4 thousand millions worldwide of whom approximately 500 million were obese (World Health Organisation 2012).

Obesity is a multifactorial complex disease influenced by lifestyle, behavioural, environmental as well as genetic factors. Obesity emanates from energy imbalance due to excess caloric intake relative to energy expenditure; the latter primarily reflects sedentary lifestyle and lack of physical activity (Wang *et al.* 2014). Furthermore, diverse evidence have shown that dysfunctional adipose tissue has an unfavourable effect on metabolism and it is related to some of the obesity associated metabolic morbidities such as IR and T2D (Sikaris 2004; Gallagher, LeRoith, and Karnieli 2010).

The accumulation of visceral adiposity, that usually occurs in obesity, has been strongly associated with insulin resistance (IR), hypertension and dyslipidaemia, and increasing rates of non-communicable chronic diseases e.g. type 2 diabetes (T2D), cardiovascular diseases (CVD) and cancer, as well as global morbidity and mortality (Sikaris 2004; Aguilera *et al.* 2008). Additionally, a decrease in protective factors mainly synthetized by the adipose tissue, such as adiponectin, and the dysregulation of inflammatory molecules could lead to a chronic low-grade inflammatory status and, later on, to metabolic syndrome (MS) (Cañete *et al.* 2007; Lankinen *et al.* 2010) (**Figure 1**). Developing new strategies for the prevention of excess weight are necessaries to tackle the rising prevalence of obesity.

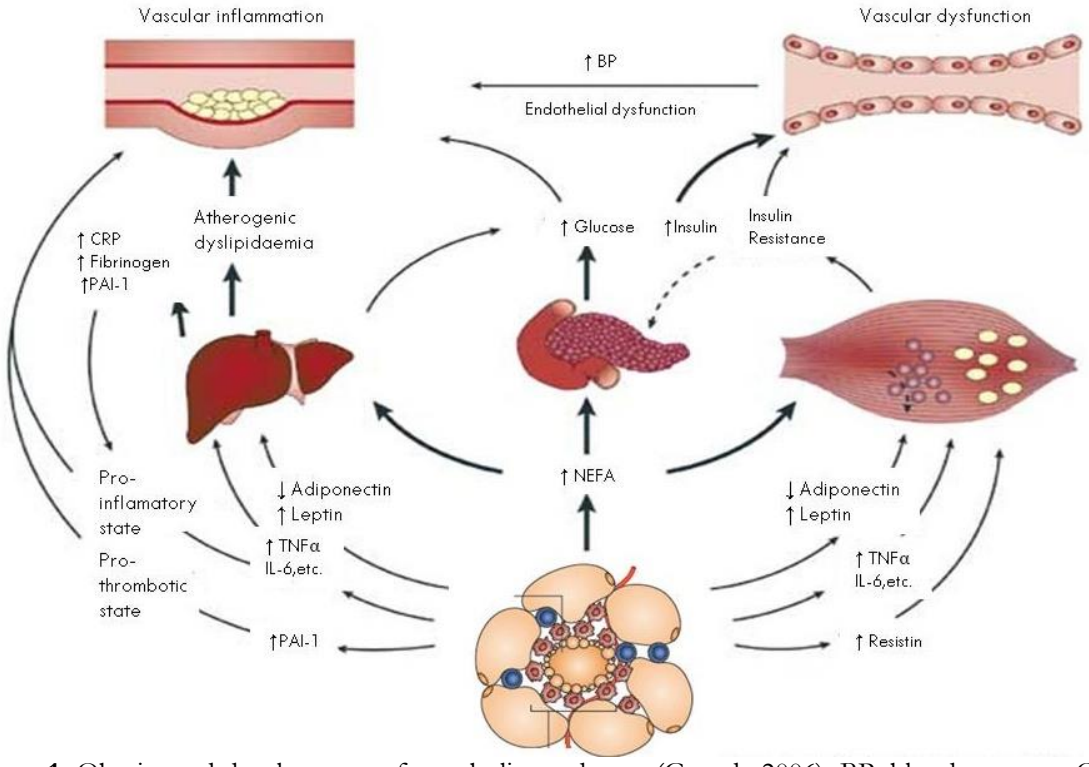

Figure 1. Obesity and development of metabolic syndrome (Grundy 2006). BP, blood pressure; CRP, C-reactive protein; IL, interleukin; TNF, tumour necrosis factor; NEFA, non-esterified fatty acids.

MS as shown on **Figure 2** is a constellation of interrelated risk factors, including disturbed glucose and insulin metabolism, obesity, dyslipidaemia, and hypertension that is associated with the development of T2D and CVD (Reaven 1988). In recent years, the prevalence of MS has increased directly with the epidemic of obesity.

According to the International Diabetes Federation (IDF) in 2005 (Alberti, Zimmet, and Shaw 2005), for a person to be defined as having the MS they must show the following criteria (**Table 1**):

Table 1. The new definition of IDF for MS (Alberti, Zimmet, and Shaw 2005).	
Central obesity (defined as waist circumference ≥ 94 cm for Caucasian men and ≥ 80 cm for Caucasian women, with ethnicity specific values for other groups) *plus* any two of the following four factors:	
Raised triacylglycerol levels:	≥ 150 mg/dl (1.7 mmol/l), or specific treatment for this lipid abnormality
Reduced High-density Lipoprotein (HDL) cholesterol	< 40 mg/dl (1.03 mmol/l) in males and < 50 mg/dl (1.29 mmol/l) in females, or specific treatment for this lipid abnormality
Raised blood pressure	Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg, or treatment of previously diagnosed hypertension
Raised fasting plasma glucose	≥ 100 mg/dl (5.6 mmol/l), or previously diagnosed type 2 diabetes. If above 5.6 mmol/l or 100 mg/dl, OGTT is strongly recommended but is not necessary to define presence of the syndrome.

BP, blood pressure; HDL, high-density lipoprotein; OGTT, oral glucose tolerance test; TAG, triacylglycerol

Increasing evidence also links inflammation and endothelial dysfunction with alterations that constitute MS. Genetics, environmental and epigenetics factors and the diet are triggers that merged with a proinflammatory state that could lead to CVD, IR and non-fatty acid liver disease.

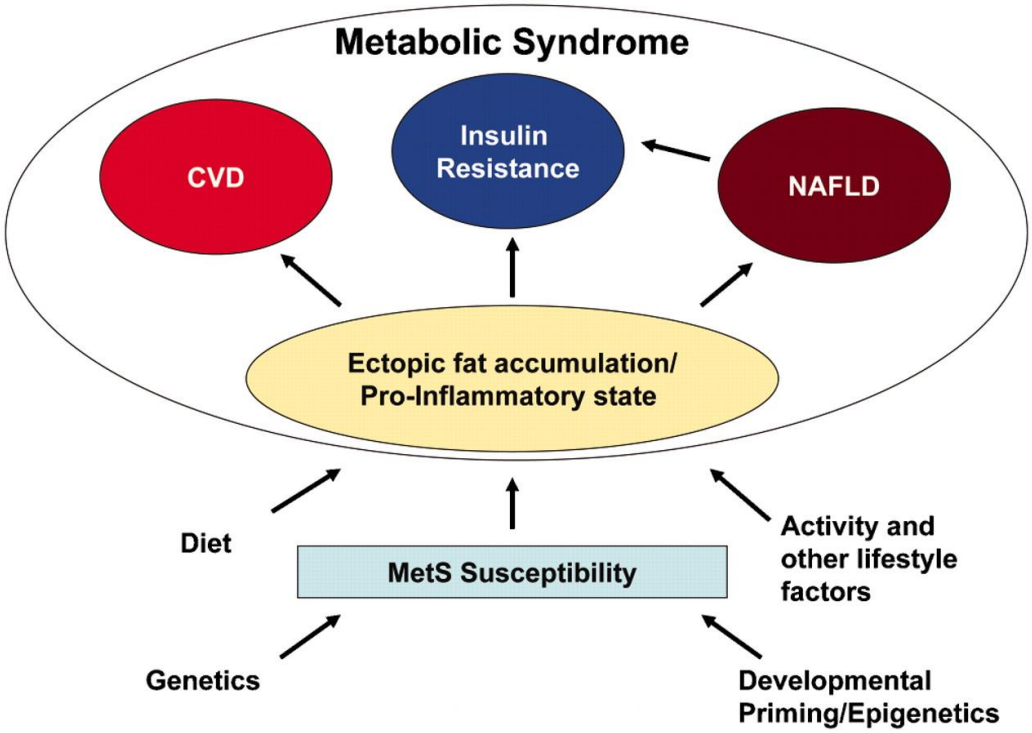

Figure 2. Factors associated with the MS. CVD, cardiovascular disease; MS, metabolic syndrome; NAFLD, non-alcoholic fatty acid liver disease.

Oxidative stress and obesity

Obesity may induce systemic oxidative stress, which is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism and its antioxidant defence systems (ADS) ROS are a variety of structures, free radicals and non-radicals, such as superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), generated by normal cellular metabolism and by exogenous agents that may serve as cell signalling or bactericidal agents. An excess of ROS formation and/or a deficient antioxidant capacity causes extensive damage in cellular macromolecules such as polyunsaturated lipids, proteins and DNA (Castilla-Cortázar 2012) (**Figure 3**).

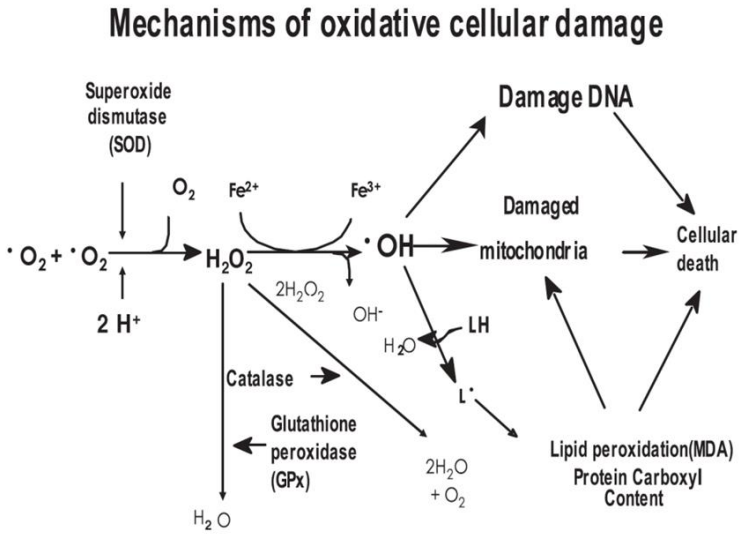

Figure 3. Representation of the formation of ROS, enzymatic antioxidant defence system and mechanism of oxidative cellular damage. (Castilla-Cortázar 2012). ROS, reactive oxygen species.

In fact, the production of ROS in adipose tissue can produce an increase in inflammation, dysregulation of adipocytokines and the migration of oxidative stress to remote tissues. Through these mechanisms, ROS contribute to the progress of IR, diabetes or atherosclerosis and developing the MS (**Figure 4**) (Fernández-Sánchez *et al.* 2011; Rupérez, Gil, and Aguilera 2014). It is well accepted that an excess of ROS enhances apoptosis or necrosis (Bernabé *et al.* 2013). Moreover, an excess of ROS production has been associated with CVD and related risk factors, such as obesity and T2D, cancer and other aging-related diseases (Choi and Lee 2010; Halliwell 2008).

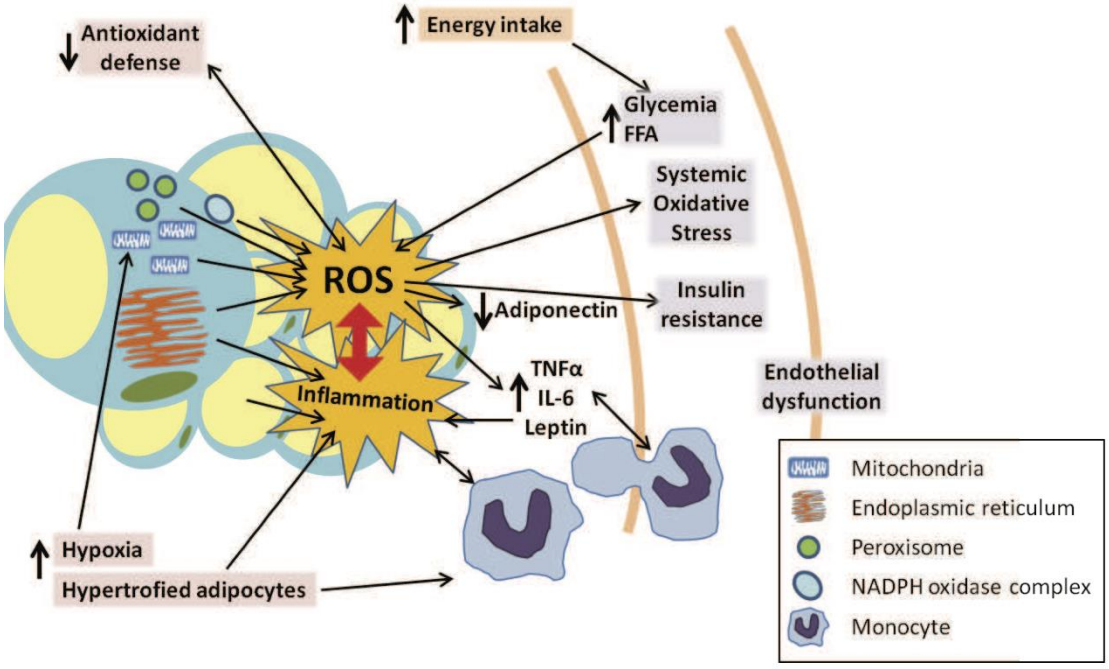

Figure 4. Linkage between obesity, oxidative stress and metabolic syndrome (Rupérez 2014). NADPH, nicotinamide adenine dinucleotide phosphate; IL-6, interleukin 6; ROS, reactive oxygen species; TNF-α, tumour necrosis alpha.

Enzymatic & non-enzymatic antioxidant mechanisms

When obesity is constant across the time, there is an unbalance in ROS production; consequently, antioxidant sources can be depleted. The **enzymatic antioxidant defence system** (E-ADS) includes, but is not limited to, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and glutathione reductase (GR).

SOD is considered a first-line defence against ROS. In humans, it is possible to find three types of SODs: mitochondrial Mn SOD, cystolic Cu/Zn. SOD catalyses the dismutation of $O_2^{\bullet-}$ to O_2 and the less reactive H_2O_2 ($O_2^{\bullet-}+O_2^{\bullet-}+2H^+ \rightarrow H_2O_2+O_2$) (McCord and Fridovich 1969). As the H_2O_2 may still react with other ROS, it needs to be degraded by either one of the other two antioxidant enzymes, catalase or GPX (Lazo-de-la-Vega-Monroy and Fernández-Mejía 2013). Catalase is a tetrameric peroxidase enzyme that converts $2H_2O_2$ to $H_2O_2 + O_2$ facilitating the reduction of organic hydroperoxides. Besides, GPX is a selenium-containing tetrameric enzyme that reduces H_2O_2 lipoperoxides and other organic hydroperoxides to their corresponding hydroxylated compounds using glutathione as hydrogen donor. Finally, GR regenerates reduced glutathione using NADPH (Roberts and Sindhu 2009) (**Figure 5**).

In vivo studies have found accumulated oxidative damage occurs from decreased levels of these enzymes rather than increased ROS production (Lazo-de-la-Vega-Monroy and Fernández-Mejía 2013). In regards, there is evidence that the activities of SOD and GPX are significantly lower in obese adults compared with normal weight people (Fernández-Sánchez *et al.* 2011). In addition, total body fat and waist circumference (WC) have been demonstrated to be positively associated with catalase levels (Silver *et al.* 2007); additionally, catalase erythrocyte activity was lower in children with insulin resistance and obesity (Rupérez *et al.* 2013; Shin and Park 2006).

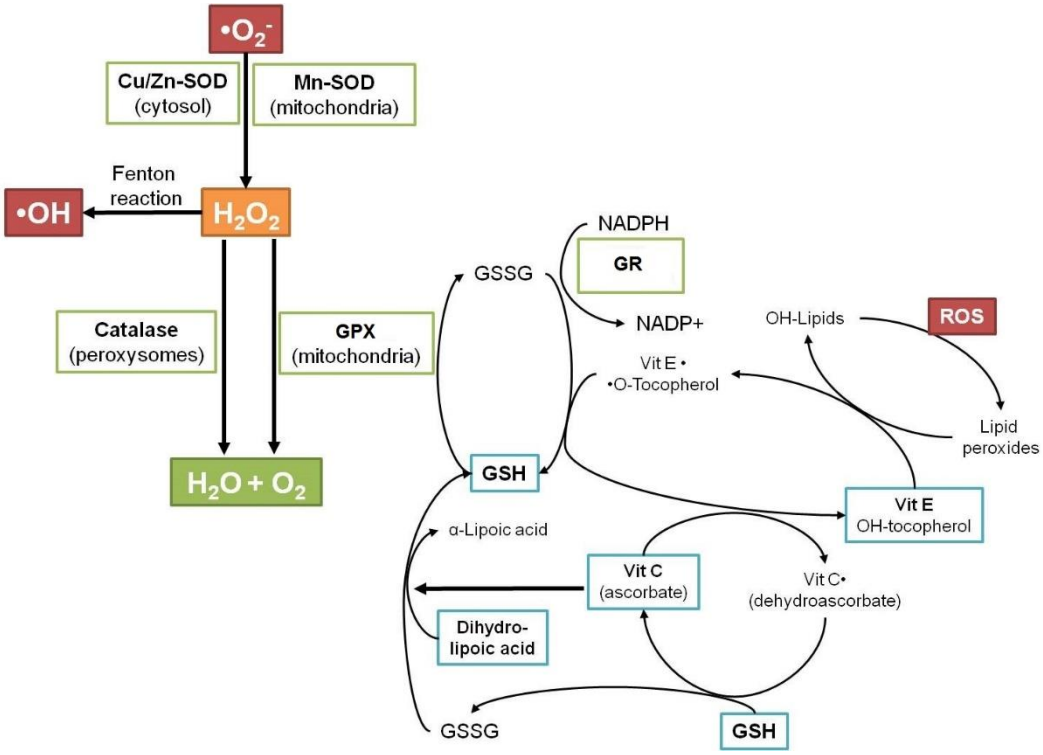

Figure 5. Enzymatic and non-enzymatic antioxidant defence systems (Lazo-de-la-Vega-Monroy and Fernández-Mejía 2013). H_2O_2, hydrogen peroxide; GPX; glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidised glutathione; GR, glutathione reductase; $O_2^{\bullet-}$, superoxide anion radical, OH, hydroxide; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; SOD, superoxide dismutase; Vit, vitamin.

In addition, there is a **non-enzymatic antioxidant defence system** (NEADS), which mostly help regenerate oxidised glutathione (GSSG) back into glutathione (GSH). Antioxidant vitamins such as A, C, E and alpha-lipoic acid are among these mechanisms (**Figure 5**). Although all these antioxidant defences work together to eliminate H_2O_2 (and thus superoxide) from the cell, in the presence of reduced transition metals (Cu, Fe), H_2O_2 can be transformed into •OH,

which is a highly reactive ROS, by the Fenton reaction (Lazo-de-la-Vega-Monroy and Fernández-Mejía 2013).

Moreover, the concentration of the NEADS, which includes the vitamin A, E, C, β-carotene are also diminished in obese subjects. (Fernández-Sánchez *et al.* 2011; Palmieri *et al.* 2006; Armutcu *et al.* 2008; Skalicky *et al.* 2008). Additionally, Li *et al.* (Li *et al.* 2013) reported that a higher serum SOD and β-carotene concentrations were associated with a lower prevalence of MS.

Oxidative stress biomarkers

The quantification of ROS is a complex challenge given the short-life nature of ROS. Nonetheless, in last years, research has been focus on the measurement of stable biomarkers that may reflect systemic oxidative stress and its degree if possible. The functional significance or the causal role of the oxidation, the specificity and the stability, the method sensitivity and reproducibility, accompanied by an appropriate biological specimen easy to be obtained, are key determinant for the validity of the biomarker. DNA, lipids, proteins and carbohydrates are examples of molecules that can be modified by excessive ROS *in vivo* and often those modified molecules can be used as biomarkers.

Lipid peroxidation

Lipids, particularly polyunsaturated fatty acids (PUFAs), are susceptible targets of oxidation (**Figure 6**) because of their reactive double bonds. In addition, PUFAs are components of cell membranes, where their side chains determine mainly the fluidity of these membranes. This characteristic is essential for the correct function of biological membranes and is deteriorate when lipids results oxidised (Willcox, Ash, and Catignani 2004).

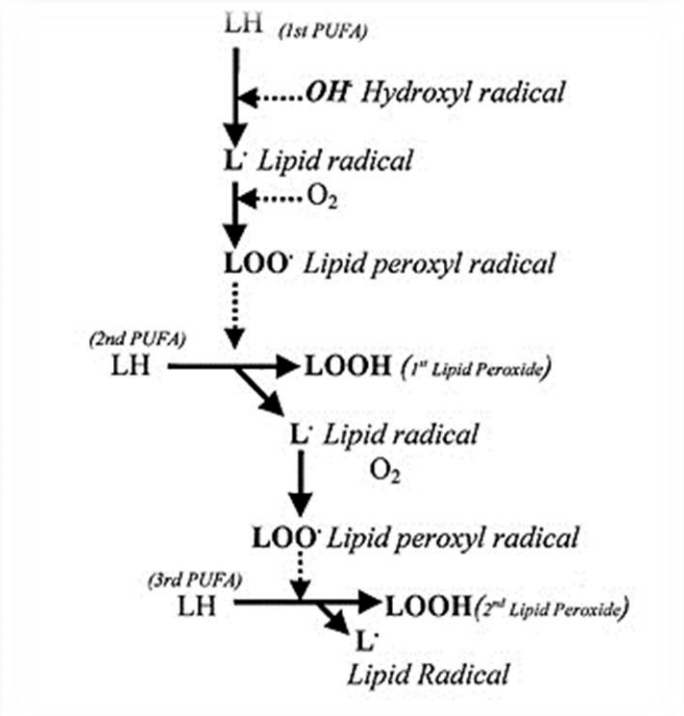

Figure 6. Lipid peroxide chain reaction. (Willcox, Ash, and Catignani 2004). $O_2^{\bullet-}$, superoxide anion radical, OH, hydroxide; LOOH, lipid hydroperoxides; PUFA, polyunsaturated fatty acid.

The F2-isoprostanes (8-iso-PGF2α) in urine and Malondialdehyde (MDA), and oxidised low-density lipoprotein (oxLDL) are examples of well-established lipid peroxidation biomarkers.

The 8-iso-PGF2α are prostaglandin-like products, produced *in vivo* primarily by free radical-induced peroxidation of arachidonic acid esterified in the *sn*-2 position of phospholipids (Petrosino and Serafini 2014). Products derived from the 8-iso-PGF2α pathway have been found to exert potential biological actions and therefore may be pathophysiological mediators of disease. Quantification of urinary 8-iso-PGF2α provides a valuable and consistent approach for assess oxidative stress *in vivo*. Elevated 8-iso-PGF2α concentration have been found in patients affected by diverse CVDs and related risk factors, such as hypercholesterolemia, diabetes, obesity and MS (Montuschi, Barnes, and Roberts 2007). Measurement of 8-iso-PGF2α is ideal in urine because it has been reported to be very stable in different circumstances, there is no significant variability between health subjects and the specimen can be obtained with non-invasive techniques (Montuschi *et al.* 2004).

On the other hand, MDA is a final product of PUFA peroxidation produced *in vivo* with potential atherogenic effects (Ho *et al.* 2013). MDA reacts with lysine residues of the apolipoprotein B (Apo-B) fractions of low-density lipoprotein leaving to oxLDL. MDA levels respond to variations in antioxidant nutrient status (Mayne 2003). Additionally, there is evidence that MDA regulates some proinflammatory and pro-atherogenic processes (Berliner and Heinecke 1996; Navab *et al.* 2004).

The major transporters of lipids in blood are low-density lipoproteins; when they are oxidised by endothelial cells and macrophages can turn into oxLDL (**Figure 7**), a molecule capable to alter coagulation processes. Oxidation of LDL is a multi-step process that begins with the initiation of lipid peroxidation by ROS/Radical Nitrogen Species within the vasculature. The peroxidation is then propagated, leading to the formation of minimally modified LDL, which is characterised by oxidation of only the lipid component. Further oxidation leads to what is referred to as oxLDL and contains both lipid and protein oxidation products. Once oxidised, the interaction between oxLDL and macrophages may be impaired and, thereby, become more atherogenic (Ho *et al.* 2013). OxLDL is involved in the origination and progression of atherosclerosis (Seifried *et al.* 2007) this particle activates circulating monocytes, increasing their ability to infiltrate the vascular wall (Willcox, Ash, and Catignani 2004). This increased infiltration is a primary stage in atherogenesis due to a stimulation of production of proinflammatory cytokines. OxLDL has shown to be a predictor of coronary artery disease in healthy subjects, since plasma concentrations are higher in patients with CVD and those increased concentration positive correlate the severity of the disease (Ho *et al.* 2013).

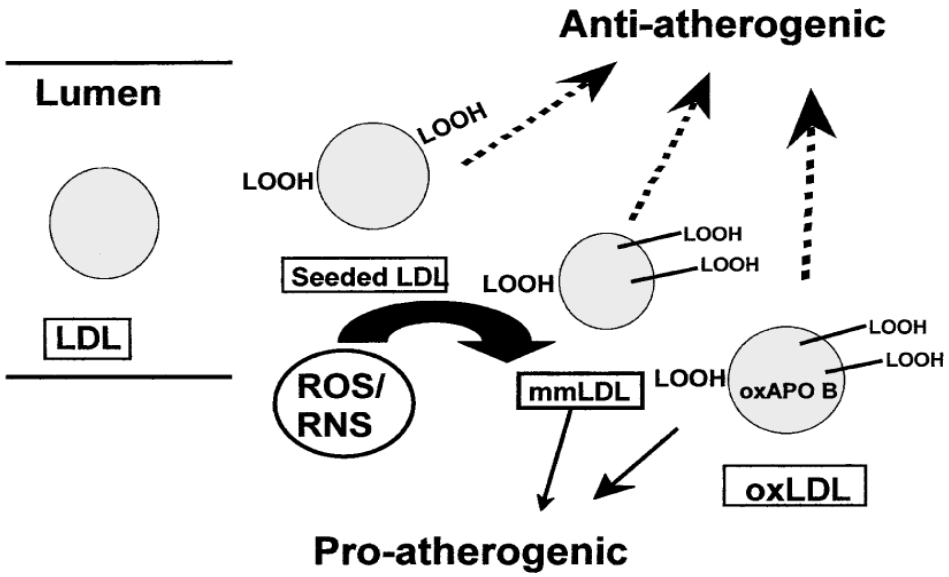

Figure 7. Pro-atherogenic effect of oxidised LDL (Ceaser *et al.* 2004). LDL, low-density lipoprotein, LOOH, lipid hydroperoxides; oxAPOB, oxidised apolipoprotein B, oxLDL, oxidised low-density lipoprotein, ROS, reactive oxygen species. RNS: Reactive Nitrogen Species; mmLDL, minimally oxidised LDL

DNA oxidation

In addition to the cytotoxic effect of lipid peroxidation, oxidative DNA damage occurs immediately and constantly. In recent years, 8-hydroxy-2'deoxiguanosine (8-OHdG) emerged as a reliable marker of oxidative stress in DNA induced by ROS, as it is a major product formed by hydroxyl radical attack to the DNA base guanine (**Figure 8**). Production of 8-OHdG takes place when DNA is being repaired from oxidative stress and is excreted in urine without further metabolism (Yao *et al.* 2004). Several studies have associated DNA damage to a wide range of aging-associated degenerative diseases such as cancer, coronary heart disease and diabetes (Wu *et al.* 2004; Valavanidis *et al.* 2009).

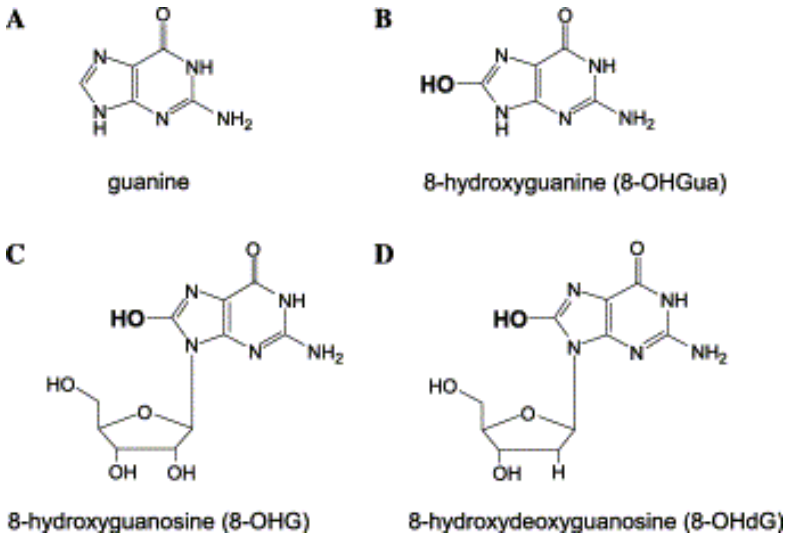

Figure 8. Chemical structure of 8-hydroxy-2'deoxiguanosine (8-OHdG) and its analogues: (A) structure of an unmodified guanine base; (B) structure of an oxidised base; (C) analogue of 8-OHdG derived from RNA; (D) structure of 8-OHdG derived from DNA. (Wu *et al.* 2004)

Inflammation and obesity

Inflammation has been recognized as a major risk factor for diverse human diseases. Inflammation is an ordered sequelae of events engineered to maintain tissue and organ homeostasis (Rangel-Huerta *et al.* 2012). It is a response to an increase of oxidative stress, which can be augmented during obesity and is characterised by vasodilation, vascular permeability and the release of inflammatory cells, i.e. neutrophils and cytokines.

Inflammation can be classified into two types: a) acute inflammation, with a rapid course (from minutes to a few days), in which the most important events are oedema and the migration of leukocytes, mainly granulocytes and monocytes; and b) chronic inflammation, characterised by a long time course, the presence of lymphocytes and macrophages and the proliferation of blood vessels and connective tissue. Hence, an exaggerated inflammatory response can cause local tissue damage and remodelling, which may induce significant and chronic injury. Inflammation underlies and/or accompanies numerous prevalent diseases, including cardiovascular diseases, obesity, diabetes, cancer, inflammatory bowel diseases, neuro-degenerative diseases, rheumatoid arthritis and asthma (Medzhitov 2008; Rangel-Huerta *et al.* 2012).

Augmented visceral adiposity, as usually takes place in obesity, is associated with a higher production of proinflammatory adipocytokines such as leptin, tumour necrosis alpha (TNF-α) and interleukins (IL) IL-1, IL-6, IL-8, and PAI-1 (Guzik, Mangalat, and Korbut 2006; Skurk and Hauner 2004). Deregulated production of these molecules participates in the pathogenesis of obesity associated MS (Medzhitov 2008).

Leptin is an adipokine secreted mainly in the white adipose tissue, and is strongly correlated with fat mass and waist circumference (Considine *et al.* 1996). Leptin plays a main role in weight control by regulating food intake and energy expenditure (Pan, Guo, and Su 2014). Leptin may have the property of lowering insulin concentration. Resistance to leptin may be connected to the hyperinsulinemia that is the earliest compensatory defect in the pathway toward the MS and glucose intolerance (Rizvi 2009).

In addition, CRP an acute phase protein, used as biomarker of low-grade inflammation, has been associated with an increased risk of T2D (Landberg *et al.* 2011) and is involved in arteriosclerosis and the development of CVD (Saito, Maruyama, and Eguchi 2015).

TNF-α is a proinflammatory cytokine produced by macrophages and endothelial cells, and has shown a great impact on the atherosclerotic process by producing metabolic perturbations, increasing the expression of adhesion molecules, surface leukocyte adhesion molecules, chemokines and enhancing the production of cytokines and growth factors (Stoner *et al.* 2013). The increase of TNF-α from the accumulated fat mass has been associated to the development of thrombosis and IR in obesity (Furukawa *et al.* 2004).

IL-1 is produced by activated macrophages as a pro-protein, which is cleaved to its active form by caspase 1. It is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. It has been shown to be a main contributor to the pathogenesis of T2D (Dinarello 2009; Esser *et al.* 2014).

IL-6 is produced by macrophages, adipocytes, fibroblasts, endothelial cells and skeletal muscle. IL-6 possess many properties ranging from defence to inflammation and it is correlated with BMI, IR and the CRP (Rizvi 2009).

IL-8 is produced by phagocytes and mesenchymal cells exposed to inflammatory stimuli and activates neutrophils inducing, chemotaxis, exocytosis and the respiratory burst. Evidence associates increases in IL-8 to obesity accompanied by hypertension and diabetes (Kim *et al.*

2006). Furthermore, it is correlated with obesity-related parameters such as BMI, WC and HOMA (Kim *et al.* 2006; Głowińska and Urban 2003; Olza *et al.* 2014).

PAI-1 acts as an important factor in the maintenance of vascular homeostasis, inhibits the activation of plasminogen and is an acute phase response protein. Studies in human adipocytes indicate that PAI-1 synthesis is upregulated by insulin, glucocorticoids, angiotensin II, some fatty acids and, most potently, by cytokines such as tumour necrosis factor-alpha and transforming growth factor-beta, whereas catecholamines reduce PAI-1 production (Skurk and Hauner 2004).

Polyphenols

Polyphenols are varieties of biological molecules and are classified according to their chemical structures (**Figure 9**), basically, phenolic acids (C6−C1 and C6−C3), flavonoids (C6−C3−C6), stilbenes (C6−C2−C6), and lignans (C6−C3−C3−C6) (Manach *et al.* 2004). Polyphenols are found in plants and are common constituents on day-to-day foods such as in wine, grapes, onions, coffee, teas, chocolate, etc. The daily intake of total polyphenols is around 1 g/d (Scalbert *et al.* 2005) being higher than most of other known antioxidants. Although researchers have attributed diverse benefits to fruit and vegetables consumption due to the high content of polyphenols and vitamins, the specific mechanisms of action remains unclear (Landete 2013).

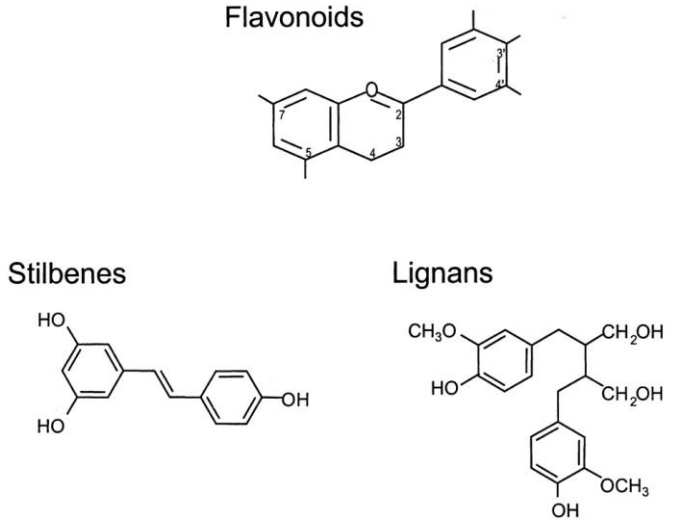

Figure 9. Chemical structure of some polyphenols (Manach *et al.* 2004)

Flavonoids

Flavonoids are secondary metabolites that may be found in fruits, vegetables and beverages derived from plants. There are several classes of flavonoids including flavanones, flavanols, flavones, flavan-3-ols, anthocyanins and isoflavones (**Figure 10**) and are classified according to their carbon structure and level of oxidation (Assini, Mulvihill, and Huff 2013). These molecules are powerful *in vitro* antioxidants with pharmacological properties such as antithrombotic and anti-inflammatory (Landberg *et al.* 2011; S. Kim *et al.* 2011). Their consumption has been shown to be inversely associated with morbidity and mortality from coronary heart diseases (Mink *et al.* 2007; Virgili and Marino 2008; Cutler *et al.* 2008) and the prevention of diverse human diseases such as cancer (Brusselmans *et al.* 2005), T2D (Wedick *et al.* 2012), neurodegenerative disorders (Zhao *et al.* 2004) and osteoporosis (Scalbert *et al.* 2005).

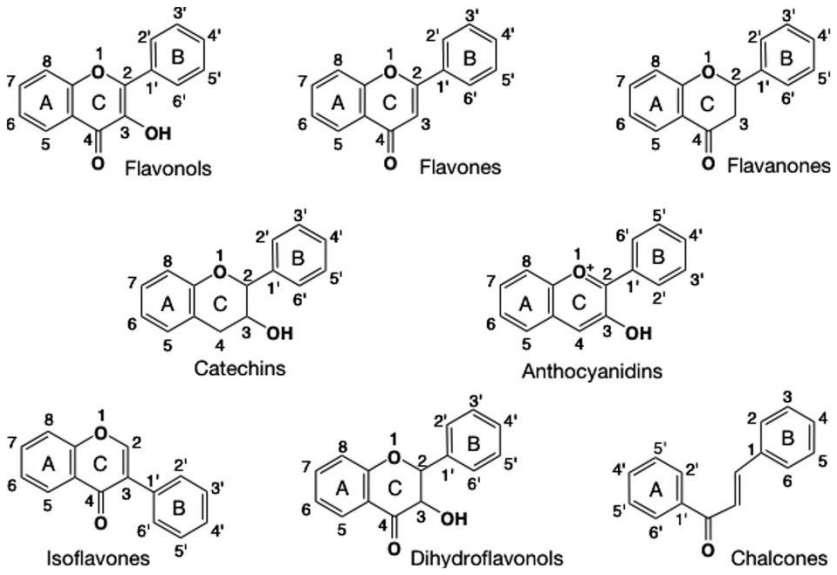

Figure 10. Flavonoids classification according to their chemical structure.

Citrus fruits contain approximately 95% of the total flavonoids encompassed mainly in flavanones, flavones and flavonols subclasses (Bahorun *et al.* 2012) (**Figure 11**).

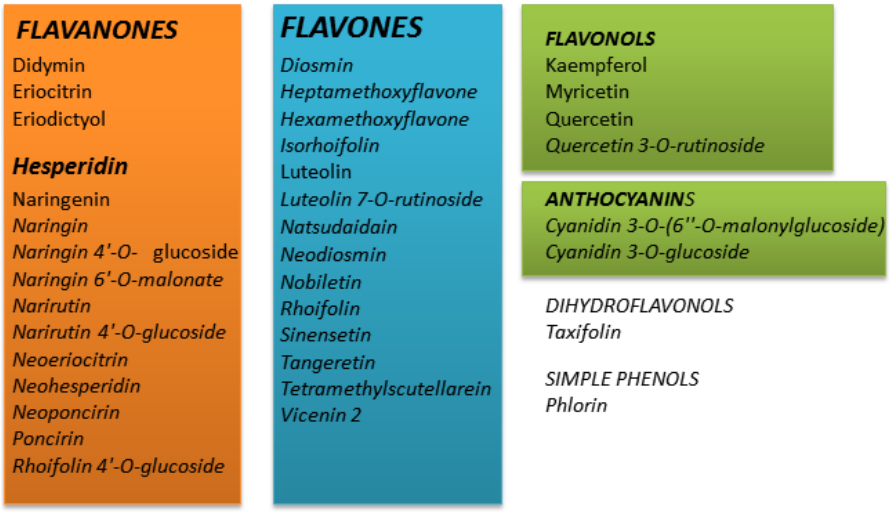

Figure 11. Flavonoids found in citrus fruits, classified by main subclasses.

Flavanones

In the past, flavanones were considered minor flavonoids, however, the discovery during the last decade of up to 350 flavanone aglycones and 100 flavanone glycosides in plants, gave them a main role in polyphenols research. Flavanones are extensively distributed in about 42 higher plant families particularly in *Compositae, Leguminosae and Rutaceae* (Khan *et al.* 2014).

Particularly, orange juice (OJ) contains the flavanones glycosides: hesperidin (200-600 mg/l) and narirutin (15-85 mg/l) (**Figure 12**).

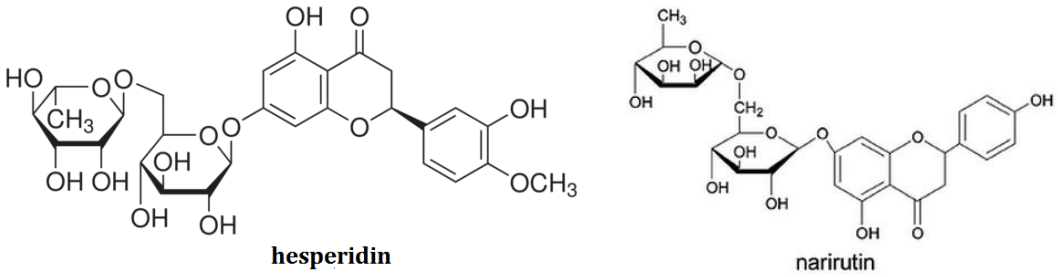

Figure 12. Chemical structure of narirutin (naringenin-7-rutinoside) and hesperidin (hesperitin-7-O-rutinoside).

The flavanones content in the solid white parts of the orange fruit is of 1360 mg/100 g in the albedo and the flavedo and 1800 mg/100 g in the membranes that separate the segments (**Figure 13**). By contrary, the pulp only contains 30 mg/100 g, explaining the high content, up to five

times, of antioxidants on whole fruit juices (Brett *et al.* 2009; Chanet, Milenkovic, Manach, *et al.* 2012; Tomás-Barberán and Clifford 2000)

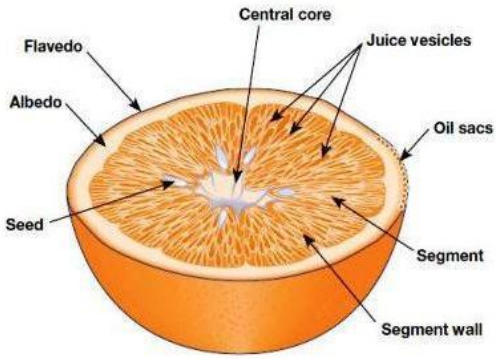

Figure 13. Orange structure. (Tetra Pak Processing Systems AB 2004).

Hesperidin (hesperitin-7-O-rutinoside) is present in high amount in lemons, limes, sweet oranges, tangerine and tangor fruits (Cano, Medina, and Bermejo 2008). In orange, hesperidin can be found in amounts between 82-324 mg/g FW in the flavedo and between 132-540 mg/g FW in the albedo. However, only in 7-27 mg/g FW can be found in the pulp (Bahorun *et al.* 2012). On the other hand, narirutin (naringenin-7-rutinoside) a glycoside from naringenin is most abundant in grapefruit but is possible to find it in significant amounts in tangor, sweet orange, tangerine and tangelo (Peterson *et al.* 2006).

Metabolism and bioavailability of flavanones

After oral intake, flavanones glycosides are hydrolysed/deglycosylated in the small intestine and colon by intestinal microbiota, producing the active aglycones hesperitin and naringin (Hertog *et al.* 1995). During the absorption, flavanones are highly modified and the released aglycones are converted into their respective glucuronides, sulphates and sulfoglucuronides during their passage across the small intestine and liver. Finally, the bioactive metabolites are distributed through plasma to different tissues and eliminated by urine; therefore, significant quantities can also be found in urinary excretions (Matsumoto *et al.* 2004). **Figure 14** summarizes flavanone digestion, absorption and metabolism.

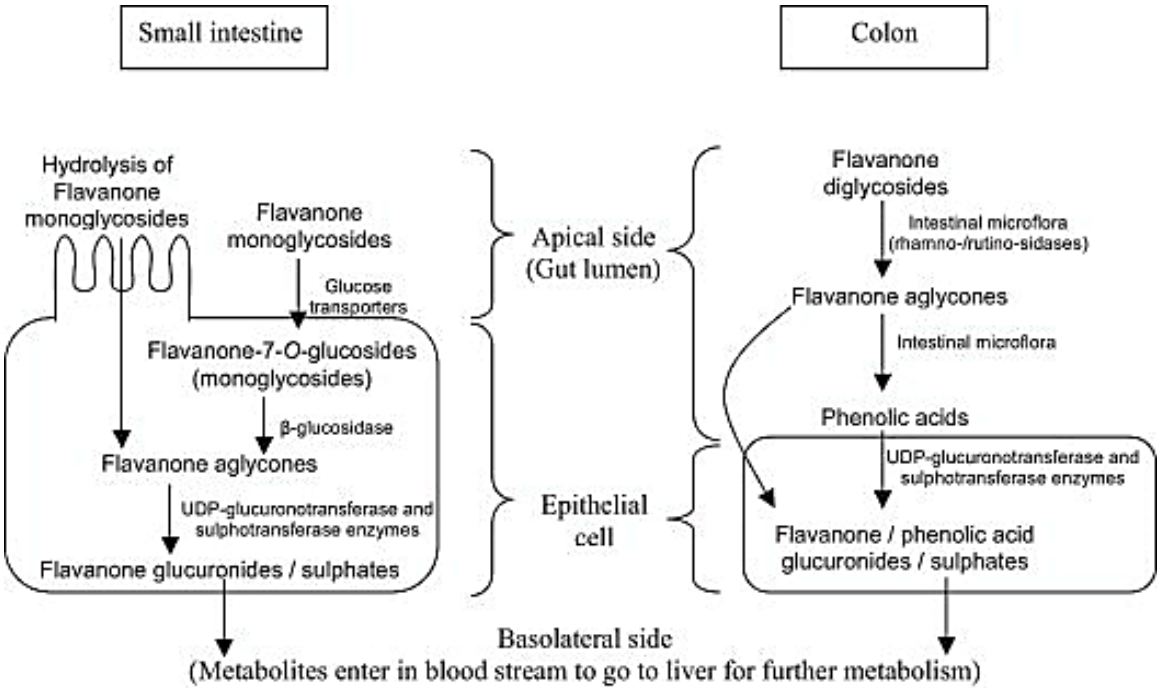

Figure 14. Flavanone digestion, absorption and metabolism (D'Archivio *et al.* 2010).

According to different bioavailability studies and evidence (Krogholm *et al.* 2010; Vallejo *et al.* 2010; Del Rio *et al.* 2010; Pereira-Caro *et al.* 2014) proposed a pathway for the hesperidin metabolism. This pathway shows that most conversions are probably mediated by colonic microbiota but some methylation steps may occur in the liver prior to excretion in urine (**Figure 15**).

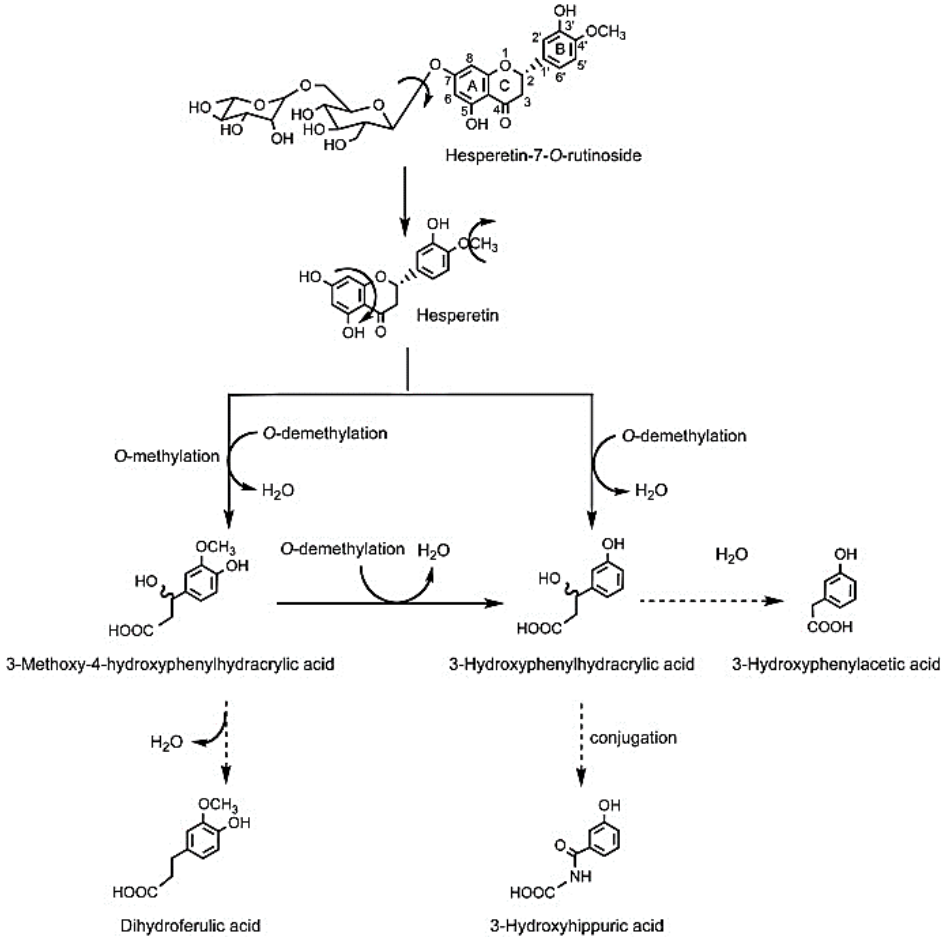

Figure 15. Proposed pathway for hesperidin metabolism (Del Rio *et al.* 2010)

Flavanones bioavailability is essential to exert its potential health benefits. A recent bioavailability study (Pereira-Caro *et al.* 2014) concluded that the excretion of hesperetin metabolites corresponded to 17.5% of the total hesperetin intake, whereas naringenin metabolites were excreted in amounts equivalent to 12.7% of ingested naringenin (see **Figure 16** for chemical structure of the main metabolites found in urine). However, when only urinary flavanone glucuronide and sulphate metabolites are analysed, flavanones, like majority of other dietary flavonoids, have only a low bioavailability. Nevertheless, when colon-derived phenolic and aromatic acids are included in the calculation, it is evident that flavanones are highly bioavailable with a recovery around ∼100% of the ingested bolus as urinary metabolites and catabolites (Pereira-Caro *et al.* 2014).

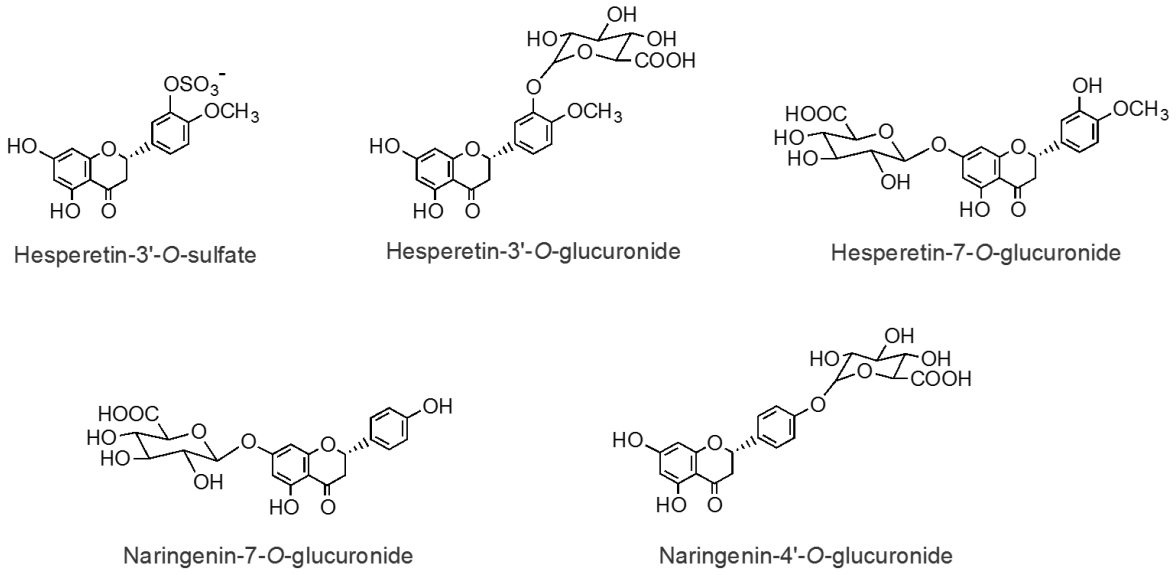

Figure 16. Structures of the main flavanone metabolites excreted in urine after orange juice consumption (Pereira-Caro *et al.* 2014)

Beneficial properties of flavanones

Different experiments *in vitro* and in animal models, had shown a reduction of inflammation biomarkers, such as CRP, TNF-α, IL-6, conducted by naringin (Lee *et al.* 2001; Choe *et al.* 2001; Chanet, Milenkovic, Deval, *et al.* 2012).

The glycosides, hesperidin and naringinin, may have anti-inflammatory, hypolipidemic, and vasoprotective properties that could lead to beneficial effects on the control of BP and LDL-cholesterol (Pérez-Jiménez *et al.* 2010; Dalgård *et al.* 2009; Scalbert *et al.* 2005). Furthermore, hesperitin and naringenin intake has been associated to a reduced risk of cerebrovascular disease (Knekt *et al.* 2002). Additionally, diverse trials had shown that doses of hesperidin >400 mg could improve the blood lipid profile, either in OJ or as a pure compound (Kurowska *et al.* 2000; Miwa *et al.* 2004; Miwa *et al.* 2005).

Recently, Buscemi and colleagues (Buscemi *et al.* 2012) demonstrated that consumption during 7 days reduced CRP and other inflammatory biomarkers such as IL-6 and TNF-α. Furthermore, other studies have reported significant reduction of the inflammation biomarker, high-sensitivity CRP after the administration of hesperidin (Dalgård *et al.* 2009; Stefano Rizza *et al.* 2011).

The consumption of citrus juices enriched in flavanones is associated with decrease of incidence in coronary heart disease, blood cells DNA oxidative damage, a decrease in Apo-B concentration (principal component of LDL-cholesterol), reduces LDL oxidability and with the improvement of plasma concentration of inflammation and vascular function biomarkers (Morand *et al.* 2011; Sharma *et al.* 2012; Sharma *et al.* 2011; Mulvihill *et al.* 2009; Wilcox *et al.* 2001; Borradaile, Carroll, and Kurowska 1999; Stefano Rizza *et al.* 2011; Miwa *et al.* 2005; Jung *et al.* 2003; Buscemi *et al.* 2012; Devaraj *et al.* 2011; Gardana *et al.* 2007; Giordano *et al.* 2011). These effects have been attributed mainly to the flavanone content of oranges and its glycosides.

Table 2 summarizes hesperidin interventions. In addition, it is still unknown how these compounds could influence the IR and the MS.

Table 2. Studies investigating the effect of hesperidin in CVD risk factors.

Author	Subjects	Intervention	Period	Significant Findings
(Rizza *et al.* 2014)	Metabolic Syndrome (n=24), 21−65 years	250 mg of hesperidin or placebo	3-wk	↑ FMD; ↓ TC, Apo-B, hsCRP
(Morand *et al.* 2011)	Healthy (n=24), 50−65 years	500 ml of CDP or CDH or OJ	4-wk	acute microvascular: ↑ for both CDH and OJ and DBP
(Demonty *et al.* 2010)	Hypercholesteraemic subjects (n=194), 18−75 years	400 mg of hesperidin or 250 mg of naringin or placebo	4-wk	No significant change on lipid profile
(Sánchez-Moreno *et al.* 2003)	Healthy (n=12), 20-32 years	500 ml/d of OJ	14-d	↓ 8-epi-PGF2α
(Ghanim, Mohanty, and Pathak 2007)	Healthy (n=72), 19-74 years	480 ml/d of OJ	8-wk	↓ IL-1 and IL-6
(Buscemi *et al.* 2012)	Obese (n=19), 18-70 years	500 ml/d of red OJ or placebo	14-d	↓ CRP, IL-6, TNF-a
(Kurowska *et al.* 2000)	Healthy (n=24), 55±1 years	250, 600 or 750 ml/d of OJ	12-wk	↑ HDL-C

CDH, control + hesperidin; CDP, control + placebo; Apo-B, apolipoprotein B; FMD, flow-mediated dilation; d, day; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IL, interleukin; OJ, orange juice; wk, week.

Metabolomics, as an innovative analytical approach

Metabolomics arose in the 1990s following the change in thought first pioneered by functional genomics that offered a more global approach to defining and understanding biology. The omics approaches provided for the investigation of interactions across the genetic, transcriptional, protein and metabolite strata; offer an alternative to other more reductionist molecular biology techniques and lea to the generation of the genomic, transcriptomic, proteomic and metabolomic fields (Roberts and Souza 2013). Metabolomics is a scientific analysis that uses a systematic pipeline of the unique chemical fingerprints present in a target organism using innovative analytical technologies. In recent years, metabolomics, has been used to discover new biomarkers and identify metabolic signatures (Meikle and Christopher 2011; Llorach *et al.* 2012; Kulkarni *et al.* 2013; Llorach *et al.* 2013).

Due to its possibility to bring a global analysis of low-molecular-weight metabolites in biological samples, metabolomics may increase understanding of human diseases and clinical risk as tracking changes in the small molecules providing a real-time estimate of disease state (Shah *et al.* 2012). The metabolic profile is characterised through the global measurement of low-molecular-weight compounds (<1,500 Da) in biological samples providing information rich-profiles.

There are three major approaches used in metabolomics. 1) Firstly, **targeted analysis**, which is a precise and quantitative measurement of the concentration of a limited number of known metabolites. 2) The **metabolic screening**, consisting of an untargeted high-throughput measurement of the levels of a large number of metabolites, including unknown metabolites. 3) Finally, the **metabolic fingerprinting**, comprising a rapid and total evaluation of biochemical patterns for discrimination of different groups in which metabolite identification is not required (Putri *et al.* 2013) (**Figure 17**).

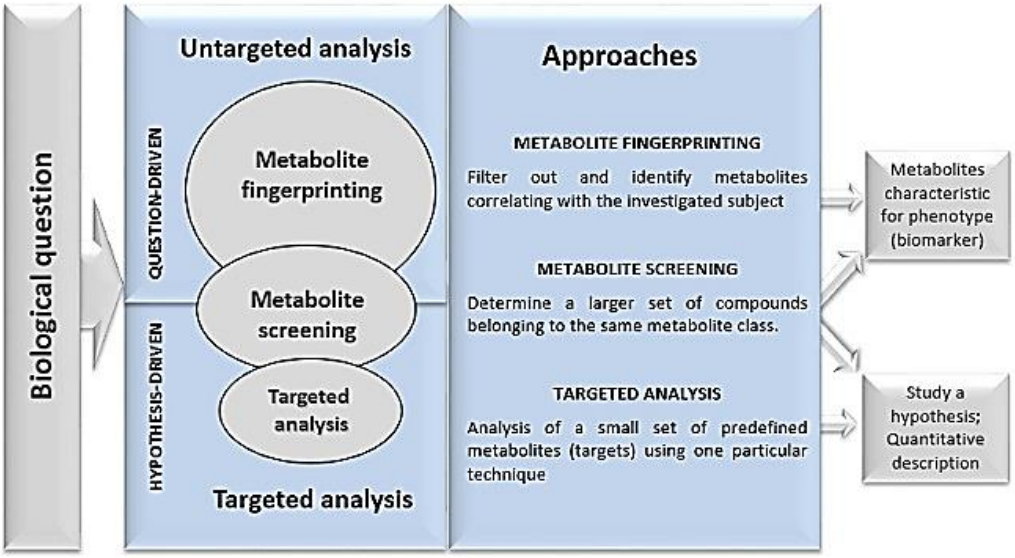

Figure 17. Different metabolomic approaches. (The Metabolomics Core Unit Biocenter Würzburg, 2015)

Different sensitive methods such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) have been used in metabolomics to detect disease biomarkers such as in obesity (Perez-Cornago *et al.* 2014; Perng *et al.* 2014), hypertension (van Deventer *et al.* 2015) and MS (Wiklund *et al.* 2014; Lin *et al.* 2014; Meikle and Christopher 2011). Moreover, in has been used to quantify biomarkers of nutrient intake and/or dietary patterns along with assessing the relationships between nutrition and the risk of disease (Catalán *et al.* 2013; Garcia-Aloy *et al.* 2015).

NMR has been used since the last decade and it is funded in measuring the specific resonance absorption profiles of metabolites in a magnetic field determined by their own chemical structure. It is characterised by the easiness of the sample preparation, non-invasiveness and non-reliance on analyte separation; hence, the samples can be used for other analysis. By contrary, is possible that the lack of sensitivity could be seen as a major disadvantage, but recently, the use of labelled compounds had improved the technique (Putri *et al.* 2013).

The GC/MS combines gas chromatography and mass spectrometry and possess advantages such as high peak capacity, excellent repeatability of retention time, and readily available compound libraries, which enable compound identification without using standard compounds. Because of the need to derivatize the samples, it is not possible to save the sample.

The combination of liquid chromatography and mass spectrometry gives the opportunity to analyse a wide range of metabolites, from low to high molecular weight, and hydrophilic or hydrophobic molecules chosen different columns (A typical workflow is shown in **Figure 18**). Additionally, the release of ultra-performance liquid chromatography (UPLC) brought a great improvement in sensitivity, shortening measurement time and high peak capacity.

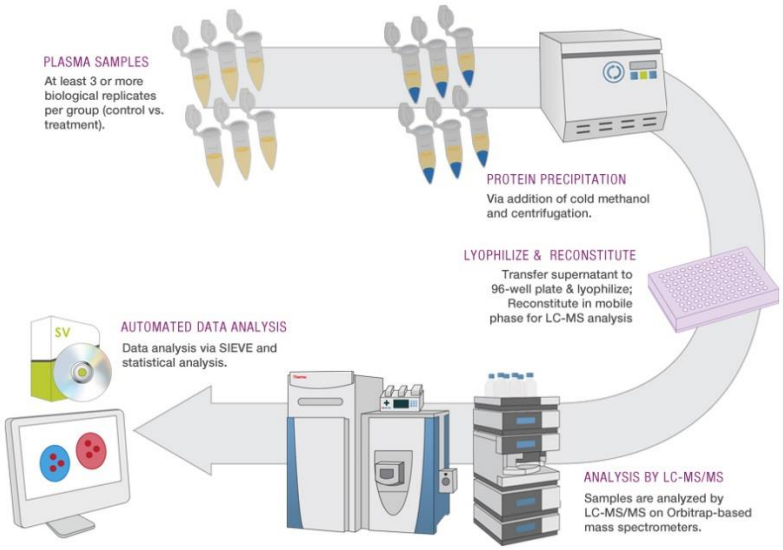

Figure 18. Typical LC-MS/MS metabolomics workflow. (Thermo Scientific, 2013)

Nevertheless, there is still opportunity for improvement in enhancing the techniques, peak capacity and stability when analysing hydrophilic metabolites simultaneously (Putri *et al.* 2013). **Figure 19** shows a comparison between analysis technologies and the sensibility brought by each one in terms of metabolites detected.

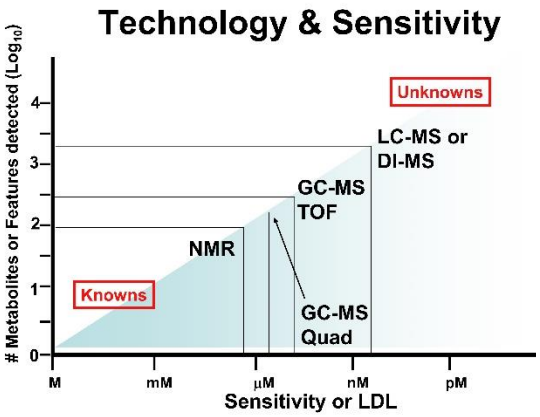

Figure 19. Sensitivity comparison between metabolomics analysis technologies. (The Metabolomics Innovation Centre, 2015). DI, direct infusion; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry, NMR, nuclear magnetic resonance; TOF, time of flight.

The high-throughput of these technologies has developed great advances in analysis of variations in the metabotype resulting from disease, food intake or dietary interventions. The possibility to capture a wide range of metabolites opens new perspectives on the discovery of new biomarkers in the nutrition research field.

Metabolomics and dietary intake

Human diets provide hundreds of phytochemicals, food additives and contaminants (known as food metabolome), it has been demonstrated that even a low exposure to some of this compounds may have an impact, beneficial or detrimental, on human health (Guertin *et al.* 2014). Many studies have observed the effects of certain dietary patterns or the inclusion of particular dietary products in health and disease; this is known as nutrimetabolomics (Guertin *et al.* 2014; Suhre 2014; Schäfer *et al.* 2014). The discovery of dietary exposure biomarkers and altered metabolites may serve as diagnostic biomarkers and enable preventive action.

Nutrimetabolomics bring the possibility to understand the inter-individual variation in response to specific nutrients and diets and the contribution of the gut microbiota to the human metabolism (Claus and Swann 2013). The influence of the food metabolome whether acutely or chronically, contributes to inter-individual variation and determines an individual's metabotype.

Frequently, Food Frequency Questionnaires (FFQ) and 24-h questionnaires has been used for assessing dietary intake but it is known that these methods tend to be inaccurate because of their subjectivity and openness to bias and under-reporting. Hence, having reliable dietary assessment methods is crucial for understanding the links between diet and chronic disease profiles. In regards, metabolomics offers a potential tool in the area of nutritional assessment, and then could fill the gap in the search of objective biomarkers of consumption and dietary patterns. For example, Heinzmann *et al.* (2010) have identified proline betaine as a putative biomarker of citrus consumption. Proline betaine is a metabolite of citrus and represents an ideal candidate biomarker, as it is metabolically inert and is therefore excreted rapidly (almost completely after 24 hours) after citrus fruit intake. Measurement of proline betaine enables a quantitative and qualitative assessment of citrus fruit intake in humans (Claus and Swann 2013). However, although nutrimetabolomics could lead to find out meaningful biomarkers to assess qualitatively and quantitatively food consumption, it is important to take in account that finding

not only a single biomarker but also a panel of biomarkers or fingerprint could increase the effectiveness in practice.

Polyphenols and metabolomics

The possibility to discover new biomarkers of polyphenol consumption represents an interesting approach for unravelling their protective effects in human health. Those protective mechanisms involves diverse regulation pathways at the molecular/cellular level as well as direct antioxidant properties. Thus, metabolomics help to identify the complex and subtle influences on whole body metabolism and physiology.

The number of studies investigating polyphenols using advanced technology had increased and different scopes has been established, i.e., analysing the consumption of phenol-rich foods and beverages, such as cocoa, wine, tea, coffee or specific polyphenols, resulted in urinary excretion of phenolic acids derived from the gut metabolism (Duynhoven *et al.* 2013; van Dorsten *et al.* 2010; Llorach *et al.* 2009; N. Khan *et al.* 2014; Garcia-Aloy *et al.* 2014; Moco, Martin, and Rezzi 2012; Luo *et al.* 2006). **Table 3** presents a brief summary of diverse suggested biological markers of polyphenolic compounds after dietary treatment in human samples.

Table 3. Summary of suggested biological markers of polyphenolic compounds after dietary treatment in human samples. Adapted from (Puiggròs *et al.* 2011)

Food/bioactive compound	N	Dietary intake assessment	Biofluid	Suggested biomarker	Time after exposure	Analytical detection method	Reference
Fruit and vegetable dietary pattern	38 subjects crossover study	300 g fruits/vegetables 2 weeks750 g fruits/vegetable 2 weeks	Urine	Naringenin, hesperidin, total flavonoids content	4–8 h	LC–APCI-MS	(Brevik *et al.* 2004)
Amanda® (almond skin extract)	16 subjects (12 + 4 placebo)	884 mg total polyphenol	Plasma	Flavan-3-ols and flavonones	2.5 h	LC–ESI-MS/MS	(Garrido *et al.* 2008)
			Urine	Epicatechin sulphate	2–6 h		
				Naringenin-O-glucoronide			
				5-(hydroxymethoxyphenyl)-γ-valerolactone	6–24 h		
				Isorhamnetin-3-O-rutinoside			
Cocoa beverage	9 subjects	42 μmol of flavan-3-ol monomers	Plasma	Epicatechin-O-sulphate	0–2 h	HPLC–PDA-MS	(Mullen *et al.* 2009)
	21 subjects		Urine	O-methyl-(epi)-catechin-O-sulphate			
				Epicatechin-O-glucoronide	0–12 h	LC–MS–MS	
				Epicatechin-O-sulphate (sum of detected)			

Table 3. Summary of suggested biological markers of polyphenolic compounds after dietary treatment in human samples. Adapted from (Puiggròs *et al.* 2011)

Food/bioactive compound	N	Dietary intake assessment	Biofluid	Suggested biomarker	Time after exposure	Analytical detection method	Reference
Pomegranate juice	28 subjects	180 ml PJ (318 mg punicalagins + 12 mg free EA (free polyphenol diet 4 days before)	Plasma	Ellagic acid	5 h	LC–MS–MS	(Seeram *et al.* 2006)
			Urine	DMEAG	12–24 h		
Black tea	3	Single dose of black tea (200 ml)	Urine	Hippuric acid (major metabolite)	0–24 h	1H NMR	(Daykin *et al.* 2005)
				1,3-Dihydroxyphenyl-2-O-sulphate (sulphate conjugate of pyrogallol)			
Black tea	53	Habitual intake	Urine	Gallic and 4-O-methylgallic acids	0–24 h	LC–MS–MS	(Mennen *et al.* 2006)
Fruits and fruit juices				Caffeic and chlorogenic acids			
Black currant and apple juice	5	4.8, 6.4, and 9.6 mg quercetin/d.	Urine	Quercetin	0–24 h	HPLC	(Young *et al.* 1999)
Orange juice, grapefruit juice	8	8 ml/kg	Plasma	Naringenin	0–24 h	HPLC	(Erlund *et al.* 2001)
			Urine				

1H-NMR, proton nuclear magnetic resonance; D; day, HPLC; high-performance liquid chromatography; ESI, electrospray ionization; LC/MS, liquid chromatography/mass spectrometry.

On the other side, metabolomics research of the benefits extended from dietary intervention with citrus polyphenols against oxidative stress and inflammation has not been carried out deeply. Llorach *et al.* (2014) had developed a study for determining citrus intake biomarkers in urine; they found proline betaine, ferulic acid and two unknown mercapturate derivatives as possible responsible of the benefits exerted by citrus juices. Nevertheless, the study of polyphenols and its metabolites in obesity and related risk factor needs to be clarified.

This comprehensive metabolite profiling approach provided an unbiased and systemic tool for the investigation of the multifactorial metabolic response following flavanones supplementation with different doses. We expected to identify biomarkers of OJ, polyphenols consumption as well as others related to oxidative stress, and inflammatory status of individuals that can be modified by the ingestion of flavanones.

AIMS OF THE STUDY

AIMS OF THE STUDY

Hypothesis

The starting hypothesis is based in the altered inflammatory and oxidative stress status presented in overweight and obese, thus, the enrichment of an OJ, a naturally container of antioxidants, with some polyphenols namely flavanones could lead to the enhancement of the inflammatory and oxidative stress status. Additionally, we were interested in investigate the effects of different doses of flavanones in the overall state.

General aims

I. The main objective was to compare two OJ enriched with different doses of polyphenols, on MS and CVD risk factors, NEADS, E-ADS and on oxidative stress biomarkers in overweight and obese volunteers.

Specific aims

I. To determine the effect of two OJs on blood pressure, glucose homeostasis, insulin, HOMA index and lipid metabolism (TAG, cholesterol, LDL-cholesterol, HDL cholesterol, Apo A1 and B) as IR and MS biomarkers.

II. To determine the influence of the two OJs on the oxidative stress status by determining urinary 8-OHdG and 8-iso-PGF2α and plasma MDA and oxLDL.

III. To determine the influence of the two OJs on the NEADS and E-ADS. For the NEADS, plasma α-tocopherol, β-carotene, retinol and Q coenzyme and glutathione. For the E-ADS, erythrocyte SOD, catalase, GPX and GR activities were determined.

IV. To evaluate the effect of the two OJs over inflammation and MS risk factors. For this purpose, leptin, IL-1β, IL-6, IL-8, TNF-α and t-PAI1 were determined.

V. To identify differential metabolites in plasma after the intake of two OJs and correlate them with biochemical, inflammation and oxidative stress biomarkers in order to identify possible biomarkers of the OJ intake, CVD, inflammation and oxidative stress biomarkers.

METHODOLOGY

METHODOLOGY

Study design

A randomised, crossover, double blind (subjects and investigators), 12-wk dietary intervention trial was conducted with OJs containing the following two different polyphenol levels: i) 0.6 mg/ml, OJ with the normal polyphenol content (NPJ) and ii) 1.5 mg/ml, OJ with the high polyphenol content (HPJ). The advantage of using a crossover design is that each subject served as its own control. A 7-wk washout period was used between the 12-wk consumption of each juice. The subjects were randomly assigned to each of the two groups, which were paired according to sex and age, using a random number generator program. The first group (n = 54) received 2 daily doses (250 ml each) of the HPJ for 12-wk (corresponding to a daily dose of 582.5 mg of hesperidin, 125 mg of narirutin and 34 mg of didymin (**Table 4**). After a 7-wk washout period, the subjects received the NPJ daily (corresponding to 237 mg of hesperidin, 45 mg of narirutin and 17 mg of didymin (**Table 4**). The second group (n = 46) received the NPJ for 12-wk followed by a 7-wk washout period, after which they received the HPJ for 12-wks (**Figure 20**). After the efficacy of the washout period was verified by confirming that the initial baseline and post-washout baseline data were similar, we merged the results from both arms of the study (the HPJ and NPJ interventions [n=100, each]) to analyse the data. Thus, our data are presented according to two periods. Period HPJ includes the data derived from all of the subjects who consumed the HPJ during the two arms of the intervention, and period NPJ includes the results obtained from all of the subjects who consumed the NPJ.

A subsample from the first arm of the study of 30 subjects, 15 of each, HPJ and NPJ groups, were chosen for a metabolomic analysis. Subjects were paired according sex and age.

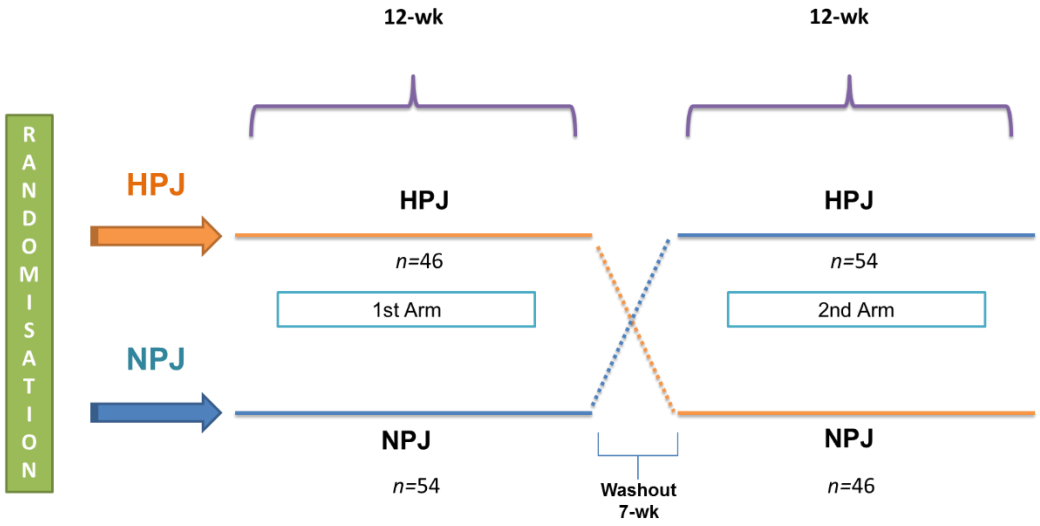

Figure 20. Randomised, crossover design. HPJ, high-polyphenols orange juice, NPJ, normal-polyphenols orange juice; wk, week.

Subject selection and allocation

The diagram of the selection, allocation and crossover randomization of the subjects involved in the study are represented in **Figure 21**. Participants were recruited through local newspaper advertisements. Among the approximately 500 volunteers who were screened, 210 (aged 18-65 years) were deemed eligible and were enrolled in the study. The sample number was estimated by considering the specific variances of the methodology for all of the variables with a type I error $\alpha = 0.05$ and a type II error $\beta = 0.2$ (80% power).

Blinding

Personnel not involved in subjects recruitment, data collection and analysis undertook the randomisation and labelling work. Participants, investigators and laboratory technicians were blinded to the treatment assignment until the conclusion of the analysis.

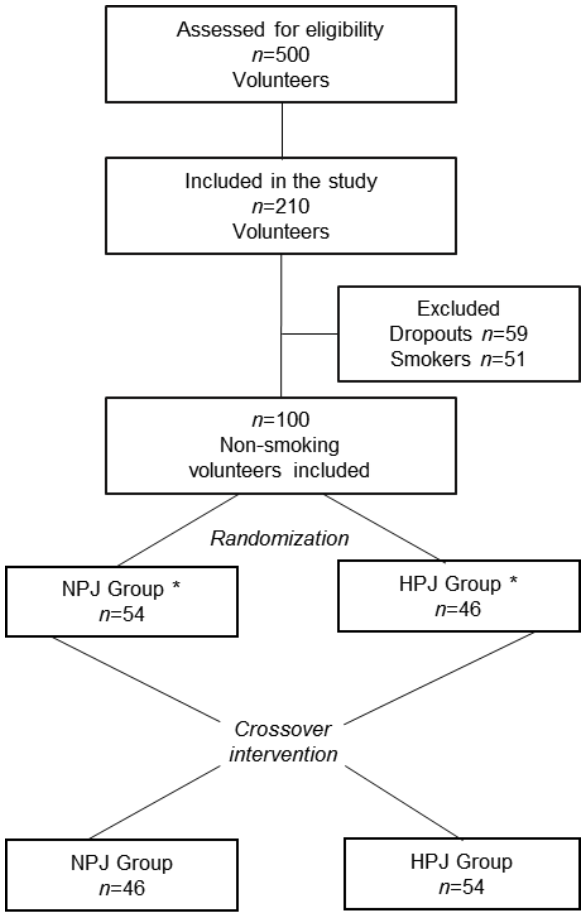

Figure 21. Flow diagram of the study. * 15 subjects were chosen for the metabolomics analysis. HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

Inclusion criteria

Inclusion criteria were BMI ≥25 (overweight or obese) but <40 (extreme obesity) or a waist circumference >94 cm for men and >80 cm for women. Most of the subjects had alterations in at least one clinical sign of MS specifically, hypertension (DBP ≥85 mmHg and <110 mmHg), hyperglycaemia (≥100 mg/dl and <130 mg/dl), elevated plasma triacylglycerol (TAG) concentrations (≥150 mg/dl), and decreased plasma HDL-C levels (<40 for men and <50 for women) as established by the IDF (Alberti, Zimmet, and Shaw 2005).

Exclusion criteria

The exclusion criteria were the presence of morbid obesity (BMI ≥40), diastolic blood pressure ≥110 mmHg, fasting plasma glucose concentration ≥130 mg/dl, and the use of any

medication for the control of blood pressure or glucose or lipid metabolism. Also a medical history of consumption of a hypocaloric diet in the last year, disorders of the gastrointestinal tract, the presence of familial dyslipidaemias in relatives of a genetic character or refusal to take part in the study.

Ethics

All study procedures were approved by the University Hospital Virgen de las Nieves Ethics Committee. The study was conducted according to the principles of the Declaration of Helsinki, and all of the volunteers gave written informed consent before the start of the intervention. This trial is registered at clinicaltrials.gov as NCT01290250.

Intervention Products

Both the NPJ and HPJ were made from fresh fruit and were provided by The Coca-Cola Europe. For the reference NPJ, a commercially available product with a normal amount of polyphenols was used (299 mg in 500 ml/d; Minute Maid®). The HPJ (Minute Maid, Whole Press®) was enriched with polyphenols that were extracted from orange albedo and pulp by a patented method and was commercially available (745 mg in 500 ml/d). Thus, the HPJ contained twice the phytocompounds of NPJ. The composition of both juices is detailed in **Table 4**. Both products were labelled to be indistinguishable one from each other and were identified as A or B.

Table 4. Composition of the OJs

	500 ml of NPJ	500 ml of HPJ
Calories, kcal	235	303
Carbohydrates, g	56.0	70.5
Fat, mg	0	0
Protein, g	5.3	5.0
Fibre, mg	2	4
Potassium, mg	460	1065
Magnesium, mg	60	65
Calcium, mg	75	105
Vitamin C, mg	210	235
Thiamine/B1, mg	0.3	0.3
Folic acid, µg	100	150
Niacin/B3, mg	1.3	2.3
Polyphenols		
Narirutin mg	45	125
Didymin, mg	17	34
Hesperidin, mg	237.5	582.5
Total polyphenols, mg	299.5	741.5

Juices were consumed twice a day in two 250 ml portions during the intervention. Values correspond to mean of the content. NPJ, normal-polyphenols orange juice; HPJ; high-polyphenols orange juice.

Study performance

Non-smoking subjects (n=100) were randomly assigned into two groups. The first group of subjects (n=54) received 2 daily doses (250 ml each) for 12 weeks of the HPJ (corresponding to a daily dose of 582.5 mg of hesperidin, 125 mg of narirutin and 34 mg of didymin). After 7 weeks of washing period, subjects received the NPJ corresponding to (237 mg of hesperidin, 45 mg of narirutin and 17 mg of didymin, daily). The other group of 46 subjects started having the NPJ for 12 weeks, followed by 7 weeks of washing and then, 12 weeks consuming the experimental beverage enriched in polyphenols. For statistical analysis, we have merged results of both groups for each treatment.

Dietary intake registration

During the time that volunteers were included in the study, they made four visits to the clinical research unit: at baseline, after the washout, and at the end of both periods of intervention. The dietary intake was performed using a FFQ and a 24-h recall (**Appendix**) at the beginning and at the end of each period. Data was managed using the CSG-software using the Spanish food composition database (http: www.bedca.net) (Martin-Moreno *et al.* 1993).

Participants were asked to maintain their usual diet and to avoid heavy physical activity, alcohol (24 h) and smoking before the visits. Once the intervention period was commenced, all volunteers were asked to consume the same calories as they were used to, in order to avoid weight gain or lost during the study. For this purpose, they received nutritional advice, so they could balance the additional calories that they were ingesting from the OJ.

Anthropometric characterisation

A single trained examiner obtained the anthropometric measurements. The participants were barefoot and wore only non-restrictive undergarments. A bioelectrical impedance analyser (Tanita Europe BV) was used to determine body weight (kg) and body composition. A scale (Anó Sanyol) was used to measure height (cm), and a flexible tape (Holtain LTD) was used to measure WC (cm). All of the measurements were obtained using standardised procedures and periodically calibrated instruments.

BP was measured according to standard methods using a validated oscillometric technique (Omron M4-I Intellisense, Omron Corporation). BP was determined in the non-dominant upper arm after a 20-min resting period. Three values were taken at 2-min intervals, and the average of these measures was considered.

Blood and urine sampling

Blood samples were collected in the fasting state between 8:00 and 11:00 am, and the samples were immediately centrifuged. Serum samples were processed at the University Hospital Virgen de las Nieves for analysis of biochemical parameters. Plasma specimens were frozen at −80°C and thawed only once for the different analysis. The erythrocytes pellet was washed three times with 0.9% NaCl (filling the tube and centrifuging at 2000 *xg* for 10 min at 4 ° C, and discarding

the supernatant each time). After washing, erythrocytes were lysed by adding cold distilled water and freezing at −80°C to ensure lysis.

First-morning urine from subjects was collected and aliquots were stored at −80 ºC until subsequent analyses.

Determination of general serum biochemical parameters

The serum levels of glucose, triacylglycerols, HDL-C, LDL-C, ApoA-I and Apo-B were measured using the clinical analysis system Roche Hitachi Modular DPP (Roche Diagnostics Spain S.L.). Fasting insulin was determined in samples using an Elecsys Modular E-170 (Roche Diagnostics Spain, S.L). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated with the equation HOMA-IR = fasting glucose (mmol/l) • fasting insulin (μU/ml)/ 22.5 (Matthews *et al.* 1985).

Determination of polyphenols in urine

The determination of both hesperetin and naringenin and their metabolites was performed using a UHPLC system, (1290 InfinitySeries, Agilent Technologies) which was equipped with a triple quadrupole mass spectrometer (6460 Series, Agilent Technologies). A Poroshell 120 C18 (Agilent Technologies; 100 x 3.0 mm i.d., 2.7 μm) was used at 30 ºC, and the injected volume was 2 μL. Gradient elution was performed using water/formic acid (99:0.1, v/v) and acetonitrile/formic acid (99:0.1, v/v) at a constant flow rate of 0.5 ml/min. The gradient commenced with the following proportions (v/v) of acetonitrile (t (min), % acetonitrile): (0, 1), (10, 40), (12, 100) and (14, 1), respectively, followed by one minute of post-time.

The optimum mass spectrometer parameters for the detection of aglycones, naringenin glucuronide (7 and 4') and hesperetin glucuronide (7 and 4') were optimized (80 μM) in negative mode, directly connecting the column inlet to the Jet Stream source. The source parameters were the same as those previously reported by Navarro *et al.* (2014).

The multiple reaction monitoring method monitored five transitions for each analysis: hesperetin, m/z 301→164; hesperetin glucuronide, m/z 477→301; hesperetin diglucuronide, m/z 653→(477)→301; hesperetin sulfoglucuronide, m/z 557→(477)→301; and hesperetin sulphate, m/z 381→301 with a dwell time for each transition of 8 ms. The concentrations of

hesperetin diglucuronide and hesperetin sulfoglucuronide metabolites were estimated by synthesized hesperetin glucuronide calibration curves (20, 10, 5, 2.5, 1 μM).

For naringenin metabolites, the reaction monitoring method transition were as follows: naringenin, m/z 271→113; naringenin glucuronide, m/z 447→271; naringenin diglucuronide, m/z 623→(447)→271; naringenin sulfoglucuronide, m/z 527→(447)→271; naringenin sulphate, m/z 351→271 with a dwell time for each transition of 8 ms. The concentrations of naringenin diglucuronide and naringenin sulfoglucuronide metabolites were estimated by synthesized naringenin glucuronide calibration curves (20, 10, 5, 2.5, 1 μM).

The limits of quantifications (LOQs) were 80, 50/80 and 40/50 nM for hesperetin, hesperetin 7 and 3' glucuronides and hesperetin 7 and 3' sulphates, respectively. The limits of detection (LODs) were 30, 20/30 and 15/20 nM, respectively. In the case of naringenin metabolites, LOQs were 30 and 70/500 nM for naringenin and naringenin 7 and 4' glucuronides, and LODs were 10 and 20/200 nM, respectively.

The intraday repeatability of the UPLC–QqQ method was assessed from ten consecutive chromatographic runs using a standard solution with 2.5 μM of every standard in MeOH: 0.1% (v/v) formic acid. The inter-day repeatability of the method was assessed by analysing the same standard solution for two consecutive days. The relative standard deviation for the peak area ranged from 0.5–4.7% for the intraday test and 1.3–3.5% for the inter-day test.

Determination of erythrocyte enzymatic antioxidant defence system activities

Determination of haemoglobin

Haemoglobin concentration in the blood samples was determined spectrophotometrically by the colorimetric cyanmethemoglobin method Drabkin (1948). Drabkin' reagent (1.0 g of sodium bicarbonate ($NaHCO_3$), 0.05 g of potassium cyanide (KCN) and 0.20 g of potassium Ferro cyanide ($K_3Fe(CN)6$, pH 7.2, Sigma Diagnostics).

Catalase activity in erythrocytes

Erythrocyte catalase activity was determined as described by Aebi (1984) and is expressed as nmol/ (L * g Hb). Briefly, after adjusting haemoglobin concentration of the original sample to 1 g/l (Drabkin 1948), 10 μl of sample was added to 90 μl of the buffer C (phosphate buffer 50

nM, pH 7.0) in a 96-well plate. Then, 50 µl of hydrogen peroxide (H_2O_2) 30 nM were added. In the ultraviolet range, H_2O_2, shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The blank is prepared with 150 µl of buffer C. the difference in absorbance per unit time is a measure of the catalase activity and is expressed as nmol/ (L • g Hb).

Superoxide dismutase activity in erythrocytes

Erythrocyte SOD activity was assayed according to the methods of McCord & Fridovich (1969). SOD catalyses the dismutation of the superoxide radical into H_2O_2. SOD activity was determined by using xanthine and xanthine oxidase to generate superoxide radicals. These radicals oxidise the cytochrome c, generating colour measured at 450 nm. The presence of SOD competes with cytochrome c in this reaction with the superoxide radical (McCord and Fridovich 1969). After adjusting haemoglobin concentration of the original sample to 1g/l (Drabkin 1948), a previous extraction of the supernatant was made with $ClCH_3$ and ethanol (1:2). Samples were centrifuged at 15600 *x g* for 3 min and 40 µl of the supernatant were mixed in 240 µl of reagent A (Ferricytochrome c 50 µM, (Horse heart cytochrome c, SOD-free, Sigma type III), xanthine 1mM, sodium carbonate buffer (20 mM pH 10.0), containing 0.1 M EDTA) into a 96-well plate and incubated for 90 seconds. The, 20 µl of reagent B (xanthine oxidase 15 mU/ml) were added and the absorbance was read at 450 nm during 3 min. One unit of SOD is defined as the amount of enzyme that reduces a 50% of spontaneous cytochrome. A calibration curve was prepared with standard solutions of SOD (0.075-15 U/ml). These results were expressed as U/mg Hb.

Glutathione reductase activity in erythrocytes

Erythrocyte GR activity was determined by Carlberg & Mannervik method (1985). The GR activity was determined by measuring the rate of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation in the presence of oxidised glutathione (GSSG). After adjusting haemoglobin concentration of the original sample to 10 g/l (Drabkin 1948), 30 µl of sample were added to 205 µl of the GSSG solution (3.7 mM, Sigma G-4376) in a phosphate buffer 147 mM with 0.47 EDTA pH 7.2, in a 96-well plate. After an incubation of 5 min at 37ºC, 40 µl of $NADPH_2$ (2.25 mM) in $NaHCO_3$ (0.1%) were added. The absorbance was monitored for another 5 min. In the ultraviolet range, NADPH shows a continual increase in absorption with decreasing wavelength, which can be followed directly at 340 nm. The blank is prepared with 30

µl of water. One unit of enzymatic activity is defined as the amount of GR that catalyses the transformation of 1 µmol of substrate NADPH per min. The results were expressed as U/g Hb.

Total activity of glutathione peroxidase in erythrocytes

Total activity of GPX activity was determined by the coupled enzyme procedure with tert-butyl hydroperoxide as substrate using the Flohé & Günzler method (1984). After adjusting haemoglobin concentration of the original sample to 10 g/l (Drabkin 1948), 25 µl of sample were added to 125 µl of phosphate buffer (100 nmol/l), 1mmol EDTA/l, pH 7.4); sodium azide 0.1M (500:10); 25 µl of enzyme glutathione reductase (2.4 u/ml, cat. No. G-4571; Sigma chemical Co., St. Louis, MO) in phosphate buffer 0.1 M, 25 u of GSH 10 mM (Sigma, G-4521) in phosphate buffer 0.1 M and 25 µl of $NADPH_2$ (1.25 mM, Sigma, N-1630) in $NaHCO_3$ (0.1%) in a 96-well plate, which was incubated 3 min at 37ºC. Then, 25 µl of tert-butyl hydroperoxide (t-BOOH, 12 mM) in water were added as start reagent. The absorbance was monitored for 5 minutes. The non-enzymatic reaction rate is correspondingly assessed by replacing the enzyme sample by buffer. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured at 37ºC. One unit of the enzymatic activity is defined as the amount of GPX that catalyses the transformation of 1 µmol of substrate NADPH per min. The results were expressed as U/g Hb.

Determination of plasma biomarkers of non-enzymatic antioxidant defence system

After extraction with 1-propanol, plasma concentrations of α-tocopherol, retinol, CoQ_9 and CoQ_{10} were determined by HPLC (1290 Infinity series, Agilent Technologies) coupled to an electrochemical detector (HPLC-EC). β-carotene was determined after extraction with 1-propanol using a HPLC system attached to a multi-wavelength ultraviolet detector set at 450 nm according to Battino *et al.* (2004). All of the compounds were identified by comparing their retention times with the predetermined retention times of the individual standards. As all of them are lipid soluble, concentrations were expressed as mg/ml of plasma.

Determination of oxidative stress biomarkers

Creatinine concentration in urine

Creatinine concentrations were determined by HPLC (1290 Infinity series, Agilent Technologies) coupled to an electrochemical detector (HPLC-EC) in CEBAS (Murcia, Spain).

Isoprostanes concentration in urine

Urinary samples were analysed for 8-iso-PGF2α through a competitive ELISA (Oxford Biomedical Research, Oxford, USA). Briefly, the 8-iso-PGF2α in the samples competes with 8-iso-PGF2α conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 8-iso-PGF2α coated on the microplate. The HRP activity results in colour development when substrate is added, with the intensity of the colour proportional to the amount of 8-iso-PGF2α-HRP bound and inversely proportional to the amount of unconjugated 8-iso-PGF2α in the samples. Then, 50 µl of standard or sample were added to microtiter plates with 50 µl of diluted 8-iso-PGF2α, incubated at room temperature for 2 h and washed by adding 300 µl of wash solution. Then, 150 µl of TMB substrate were added and allowed to react at room temperature until colour changed. The reaction was stopped with 50 µl of a solution of 3 M sulphuric acid. Absorbance of each well was read at 450 nm. The determination range was 0-75 ng/ml and a CV of 7.2%. Concentration was normalized using urinary creatinine, calculating the 8-iso-PGF2α /Creatinine ratio and expressed in ng/mg of creatinine.

8'dehydroxyguanosine concentration in urine

Levels of urinary 8-OHdG were determined by a competitive ELISA kit (JAICA, Fukuroi, Japan). In brief, 50 µl of primary monoclonal antibody and 50 µl of sample or standard were added to microtiter plates, which were pre-coated with 8-OHdG, incubated at 37ºC for 1 h and washed with 250 µl of phosphate-buffered saline (PBS). Then, 100 µl of HRP-conjugated secondary antibody was then added to each well, incubated at 37 ºC for 1 h and washed with 250 µl of PBS. Then, 100 µl of enzyme substrate was then added to each well and allowed to react at room temperature for 15 min. The reaction was terminated with 100 µl of 1 N phosphoric acid. Absorbance of each well was read at 450 nm by a microplate reader. The determination range was 0.125-10 ng/ml and a CV of 8.3%. Concentration was normalized using

urinary creatinine, calculating the 8-OHdG/Creatinine ratio and expressed in ng/mg of creatinine.

Oxidised LDL concentration in plasma

A commercial ELISA kit was (Biomedica, Wien, Austria) was used to determine oxLDL. Briefly, 100 µl of sample or standards were added to the wells and incubated at 37 ºC during 2 h, after being washed 100 µl of anti-oxLDL antibody was added to each well and incubated another 1 h at 37ºC. Then, 100 µl of substrate were added into each well and incubated in dark at room temperature for 30 min, next 50 µl of the stop solution were added and the absorbance was measured spectrophotometrically at 450 nm in a plate reader (Synergy HT, Biotek Instruments). The determination range was 0 to 750 ng/ml and a CV of 11.8%.

Lipid peroxides concentration in plasma

A colorimetric commercial assay kit (Oxystat, Biomedica; Vienna, Austria) was used to determine the total LPO concentration in EDTA-plasma samples (detection limit, 7 µmol/l; CV 8.3%). Peroxide concentration was determined by adding 10 µl of sample and 100 µl of sample buffer and a subsequent colour-reaction using 3, 3', 5, 5'-tetramethylbenzidine as substrate. After addition of 50 µl sulphuric acid as stop solution, the coloured liquid was measured photometrically at 450 nm in a plate reader (Synergy HT, Biotek Instruments). A calibrator was used to calculate the concentration of circulating biological peroxides in the sample, with a detection limit of 7 µmol/l.

Malondialdehyde concentration in plasma

MDA was determined in plasma using a thiobarbituric acid reactive substances (TBARS) assay kit (OxiSelect; Cell Biolabs, Inc.; Sand Diego, USA). 100 µl of sample or standards was added to 100 µl of a SDS lysis solution and incubated at room temperature for 5 min. 250 µl of TBA reagent was added to each well and incubated at 95 ºC during 1 hour. After cooling at room temperature during 5 min and centrifuged for 15 min, was absorbance was measured spectrophotometrically at 532 nm in a plate reader (Synergy HT, Biotek Instruments). The determination range was 0-125 µM and a CV of 2.15%

Determination of adipokines and inflammation biomarkers in plasma

Affymetryx human monoclonal antibody kits (Affymetryx, Santa Clara, CA) were used according to the manufacturer's protocols using the Luminex 200 system (Luminex corp., Austin, TX) built on xMAP technology to determine concentrations of the following biomarkers: adipokines (leptin) and inflammatory (IL-1, IL-6, IL-8, TNF-α and tPAI-1). The xMAP technology allows for detection and analysis of up to 100 analytes per well of a 96-well plate. This technology combines fluidics, optics and digital signal processing. Luminex colour-codes tiny beads, called microspheres, into 500 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Inside the Luminex analyser, a light source excites the internal dyes that identify each microsphere particle, and any reporter dye captured during the assay. Many readings are made on each bead set, which further validates the results (**Figure 22**).

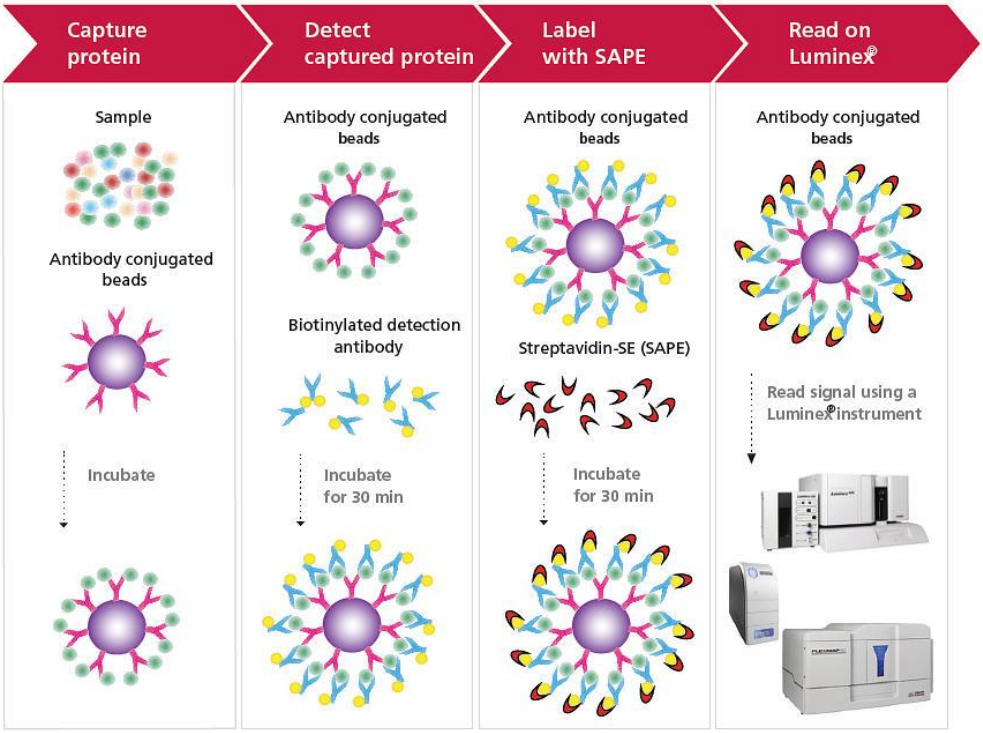

Figure 22. xMAP technology standard protocol built on Luminex reader.

Statistical analysis

Values are presented as the mean ± SEM. Prior to the statistical analyses; the data were assessed for normality using the Shapiro-Wilk test. The homogeneity of the variances was estimated using the Levene's test. An independent samples *t*-test was conducted to identify possible differences for the study variables between the groups that started with either NPJ (n= 46) or HPJ (n = 54) at baseline. To verify the efficacy of the washout period, a paired *t*-test was conducted between the initial baseline data and the post-washout baseline data. Once the efficacy of the washout period was verified, the initial baseline and post-washout baseline data were combined, and the final data from each study arm (n=100 each for the two interventions) were combined. Then, paired-sample *t*-tests were conducted between the baseline and post-intervention data to determine the effects of each intervention on each parameter.

A linear mixed-effects model (LMM), with the intercept as random effect and a covariance structure for repeated measures by time and OJ, was used to determine the differences between the interventions (i.e., intervention effects). This model allows for the heterogeneity of the outcome responses between juice intake at each time point and at the individual level of the participants. Note that the baselines were also treated as outcome responses but without the associated juice intake effects. Therefore, in the model, time was included as a categorical variable indicating the beginning and end of each arm of the trial. Furthermore, the fixed effects included time, juice intake, age, gender, BMI, WC, energy intake, and the interaction between juice intake and the arms of the intervention (i.e., P-interaction), and they were used as covariates to adjust for possible confounding factors. Energy intake did not have an impact on any variable, thus, was eliminated from the models.

Correlations between the concentrations of the main flavanones and variables were estimated by the Pearson's correlation coefficient when the assumptions of normality were met and by the Spearman's correlation coefficient when the assumptions of normality were not met. $P < 0.05$ was considered as significant. The correlations were obtained using the delta (baseline – post-intervention) value of both groups pooled to increase the n and in order to evaluate the effect of doses of flavanones. All of the statistical analyses were performed using SPSS 20.0 (IBM Corp.) for Windows.

Metabolomics approach

Subjects

A subsample of 30 subjects from the first arm of the intervention, aged between 22-63 y were selected. Subjects were chosen according to percentage of compliance to the intervention and paired by sex and age.

Global biochemical profiles were determined by Metabolon Inc. (NC, USA), in human serum, representing gender and age matched intervention groups collected at initial baseline and final time points, as detailed in **Table 5.**

Table 5. Groups of distribution for metabolomics approach

Group	*n*		Description
	Baseline	Final	
NPJ	15	15	NPJ consumption (8 female, 7 male)
HPJ	15	15	HPJ consumption (7 female, 8 male)

HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice

Sample Preparation

Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. A recovery standard was added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) and then centrifuged. The resulting extract was divided into five fractions: one for analysis by UPLC-MS/MS with positive ion mode, electrospray ionization, and one for analysis by UPLC-MS/MS with negative ion mode, electrospray ionization, one for LC polar platform, one for analysis by GC-MS, and one sample was reserved for backup. Samples were placed briefly on a TurboVap ® (Zymark, California, USA) to remove the organic solvent. For LC, the samples

were stored overnight under nitrogen before preparation for analysis. For GC, each sample was dried under vacuum overnight before preparation for analysis.

Quality controls

Several types of controls were analysed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate throughout the data set extracted, and water samples served as process blanks. A cocktail of quality control (QC) standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analysed sample, allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Entire process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomised across the platform run with QC samples spaced evenly among the injections, as outlined in **Figure 23**. A small aliquot of each project sample (coloured cylinders) is pooled to create a control matrix (CMTRX) technical replicate sample (multi-coloured cylinder), which is then injected periodically throughout the platform run. Variability among consistently detected biochemicals can be used to calculate an estimate of overall process and platform variability.

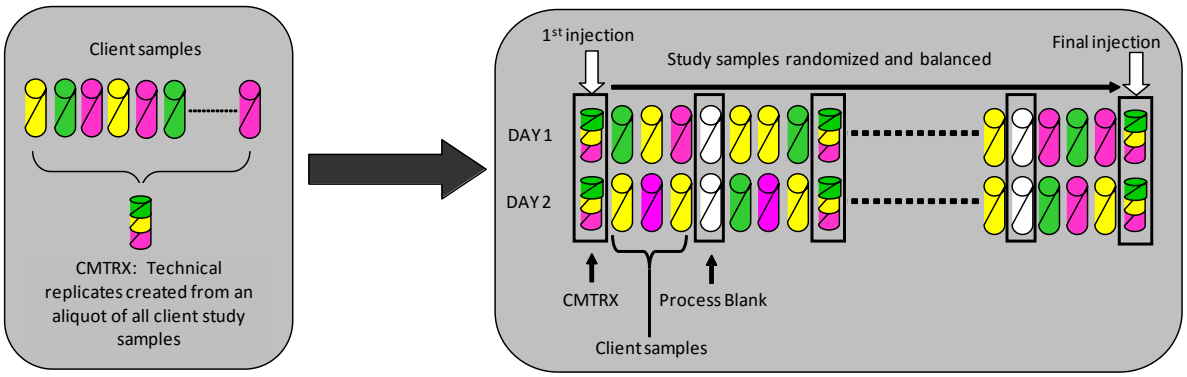

Figure 23. Preparation of project-specific technical replicate quality controls.

Analytical platforms

Ultra-performance liquid chromatography tandem mass spectroscopy

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyser operated at 35,000 mass resolution. The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained eight or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analysed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm). Extracts reconstituted in acidic conditions were gradient eluted from a C18 column using water and methanol containing 0.1% formic acid. The basic extracts were similarly eluted from C18 using methanol and water, however with 6.5 mM ammonium bicarbonate. The third aliquot was analysed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion, and the scan range was from 80-1000 m/z.

Gas chromatography/Mass spectrometry

The samples for analysis by GC-MS were dried under vacuum for a minimum of 18 h prior to being derivatized under dried nitrogen using bistrimethyl-silyltrifluoroacetamide. Derivatized samples were separated on a 5% diphenyl/95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 um film thickness) with helium as carrier gas and a temperature ramp from 60° to 340 °C in a 17.5 min period. Samples were analysed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power. The scan range was from 50–750 m/z.

Bioinformatics

Data extraction and compound identification

Raw data was extracted, peak-identified and QC processed using specific Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 0.005 amu, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

Curation

A variety of curation procedures were carried out to ensure that a high quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artefacts, mis-assignments, and background noise. Metabolon's data analysts used proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

Metabolite quantification and data normalization

Peaks were quantified using area-under-the-curve. Data normalization step was performed to correct variation resulting from instrument differences. Essentially, each compound was corrected by registering the medians to equal one (1.00).

Statistical analysis

Fold Change

Fold change (FC) is a measure describing how much a quantity changes going from an initial to a final value. In the present study two FCs were calculated, one comparing the baseline *vs* final time point (Final/Baseline) of each experimental group and another one comparing the NPJ *vs* HPJ (HPJ/NPJ) group at baseline and final time points. The benefit of using the FC regards to the use of ratio between to values, which emphasizes itself the change rather than absolute values. To understand FC, values > 1 means an increase, while values < 1 means a decrease.

*S*tudent *t*-test

A *t*-test for independent samples was carried out identify possible differences at baseline between groups. Moreover, a *t*-test for equality of means between baseline and post-intervention in each group and for each study variable was carried out to determine changes after each intervention.

Analysis of variance

For the analysis of variance (ANOVA), it is assumed that all populations have the same variances. One-way ANOVA is used to test whether at least two unknown means are all equal or whether at least one pair of means is different. For the case of two means, ANOVA gives the same result as a two-sided *t*-test with a pooled estimate of the variance. Three types of effect were determined*, time (basal vs final), intervention (NPJ vs HPJ) and time x intervention* interaction.

Statistical significance

The *P*-value is the probability that the test statistic is at least as extreme as observed in this experiment given that the null hypothesis is true. The false discovery rate (FDR) is the expected proportion of erroneous rejections among all rejections when applying a multiple test comparison. There are different methods to correct for multiple testing. The oldest methods are familywise error rate adjustments (Bonferroni, Tukey, etc.), but these tend to be extremely conservative for a very large number of tests. The familywise error rate adjustments give one a high degree of confidence that there are zero false discoveries. However, with FDR methods, one can allow for a small number of false discoveries. The FDR for a given set of compounds

can be estimated using the q-value (Storey and Tibshirani 2003). Q-values are the name given to the adjusted p-values found using optimized FDR approach.

In order to interpret the q-value, the data must first be sorted by the p-value then choose the cut-off for significance (typically $P < 0.05$). The q-value gives the false discovery rate for the selected list (i.e., an estimate of the proportion of false discoveries for the list of compounds whose p-value is below the cut-off for significance). For **Table 6** below, if the whole list is declared significant, then the false discovery rate is approximately 10%. If everything from Compound 079 and above is declared significant, then the false discovery rate is approximately 2.5%. In the present study, we chose a cut-off for significance of $q < 0.1$.

Table 6: Example of q-value interpretation

Compound	*p* -value	*q* -value
Compound 103	0.0002	0.0122
Compound 212	0.0004	0.0122
Compound 076	0.0004	0.0122
Compound 002	0.0005	0.0122
Compound 168	0.0006	0.0122
Compound 079	0.0016	0.0258
Compound 113	0.0052	0.0631
Compound 050	0.0053	0.0631
Compound 098	0.0061	0.0647
Compound 267	0.0098	0.0939

Correlations

Correlations between the concentrations of the main flavanones and the median scaled intensity of the metabolites detected were estimated by the Pearson's correlation coefficient when the assumptions of normality were met and by the Spearman's correlation coefficient when the assumptions of normality were not met. $P < 0.05$ was considered as significant. The correlations were obtained using the delta (baseline – post-intervention) value of both groups pooled to increase the n and in order to evaluate the effect of doses of flavanones

Box-and-whisker plots

Box-and-whisker plots is a histogram-like method of displaying data designed by Tukey (1977). This plot includes five descriptive measurements from a dataset: the median, the first and third quartiles, the minimum and the maximum value. Furthermore, in the boxplot presented here, the mean is also included. Additionally, it is possible to identify outliers from the

data set. For a better understanding, interpretation, **Figure 24** is an example of how the data displays in the graphics, and the box plot legend explains each part of the plot.

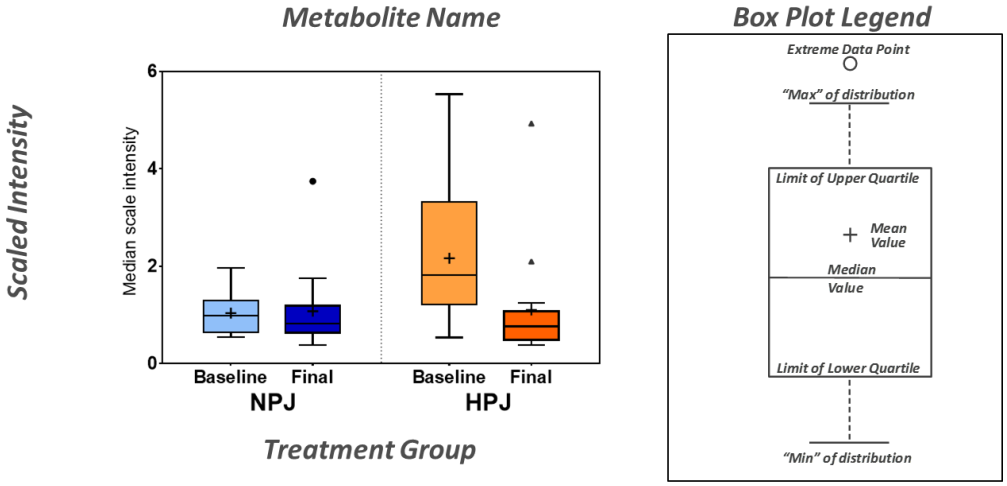

Figure 24. Example of data display on box-and-whisker plots. The graphic shows the comparison in each group at baseline and final time. HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

Principal Component Analysis

The first objective in the data analysis process was to reduce the dimensionality of the complex data set to enable easy visualization of any metabolic clustering of the different groups of samples. This has been achieved by a principal component analysis (PCA) where the data matrix is reduced to a series of principal components (PCs), each a linear combination of the metabolite peak areas. The number of principal components is less than or equal to the number of original variables. This transformation is defined in such a way that the first principal component has the largest possible variance (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it is orthogonal to (i.e., uncorrelated with) the preceding components. Two types of plots were used: 1) Scores plot displays the samples as situated on the projection planes described by the PCs; 2) Loadings plot shows the influence of the metabolites on clustering appearing in the scores plot. The loadings plot, which indicates the spectral variables responsible of the patterns and trends found, facilitates interpretation of the scores plot: as metabolites with different levels between two groups appear in the same region of the scores

and loadings plots, the comparison between plots enables to understand how the metabolites relate to the samples.

The number of components or model dimensions (A) included in the model is linked to the difference between the degree of fit and the predictive ability (R^2 and Q^2 parameter respectively). R^2 provides an indication of how much of the variation within the data set can be explained by the model (goodness of fit). Q^2 parameter describes the predictive ability of model (goodness of prediction). The R^2 and Q^2 parameters display entirely different behaviour as the model complexity increases (A increases). The goodness of fit, R^2, varies between zero and 1, where 1 means a perfectly fitting model and zero no fit at all. However, R^2 is inflammatory and approaches to unity as A increases. Hence, it is not sufficient to have a high R^2. On the other hand, the goodness of prediction, Q^2, is less inflammatory and will not automatically come close to 1 with increasing A. Additionally, depending on the type of samples included in the analysis the cut-off values might change, but usually if Q^2 value is over the 20% of R^2 it can be consider as a good PCA model.

Random Forest

Random forest (RF) is a supervised classification technique based on an ensemble of decision trees (Breiman 2001). For a given decision tree, a random subset of the data with identifying true class information is selected to build the tree ("bootstrap sample" or "training set"), and then the remaining data, the "out-of-bag" (OOB) variables, are passed down the tree to obtain a class prediction for each sample. This process is repeated thousands of times to produce the forest. The final classification of each sample is determined by computing the class prediction frequency ("votes") for the OOB variables over the whole forest. For clarifying, supposing that the RF consists of 50,000 trees and that 25,000 trees had a prediction for sample 1. Of these 25,000, suppose 15,000 trees classified the sample as belonging to Group A and the remaining 10,000 classified it as belonging to Group B. Then the votes are 0.6 for Group A and 0.4 for Group B, and hence the final classification is Group A. This method is unbiased since the prediction for each sample is based on trees built from a subset of samples that do not include that sample. When the full forest is grown, the class predictions are compared to the true classes, generating the "OOB error rate" as a measure of prediction accuracy. Thus, the prediction accuracy is an unbiased estimate of how well one can predict sample class in a new data set. RF has several

advantages: it makes no parametric assumptions, variable selection is not needed, it does not over fit and it is invariant to transformation.

To determine which variables (metabolites) make the largest contribution to the classification, a "variable importance" measure is computed. The "Mean Decrease Accuracy" (MDAC) is used as this metric. The MDAC is determined by randomly permuting a variable, running the observed values through the trees, and then reassessing the prediction accuracy. If a variable is not important, then this procedure will have little change in the accuracy of the class prediction (permuting random noise will give random noise). By contrast, if a variable is important to the classification, the prediction accuracy will drop after such a permutation, which we record as the MDAC. Thus, the RF analysis provides an "importance" rank ordering of biochemicals; here are presented the top 30 biochemicals as potentially worthy of further investigation.

RESULTS

RESULTS

Pre-intervention characteristics of subjects by groups

Before the intervention, the anthropometric, body composition, and biochemical parameters were similar between the two groups of volunteers, with the exception of DBP ($P = 0.049$), as shown in **Table 7.**

Table 7. Anthropometric characteristics and body composition, serum biochemical parameters and urine polyphenols in overweight and obese adults at baseline before the HPJ and NPJ interventions.

	NPJ	HPJ
Weight, kg	93.3±2.5	91.4±1.9
BMI, kg/m^2	33.1±0.6	33.2±0.5
WC, cm	105±2	102±1
SBP, mm Hg	132±2	129±2
DBP, mm Hg	83±1	79±1$^{\#}$
Body water, kg	43.1±1.3	41±1.1
% Water	46.2±0.7	44.7±0.7
Body fat, kg	34.1±1.4	34.7±1.2
% Body fat	37.5±1.3	37.8±1
Lean mass, kg	59.1±1.6	56.9±1.5
Muscle mass, kg	55.8±1.6	54.1±1.4
Glucose, mmol/l	4.8±0.1	4.7±0.1
Insulin, µU/ml	14.9±1.2	15.7±1.4
HOMA-IR	3.3±0.3	3.5±0.4
TC, mg/dl	211±5	217±5
HDL-C, mg/dl	49±2	50±2
LDL-C, mg/dl	127±4	133±4
TAG, mg/dl	142±9	137±9
ApoA-I, mg/dl	147±3	147±3
Apo-B, mg/dl	96±3	100±3
Urine hesperidin, mg/l	0.83±0.36	1.10±0.41
Urine naringenin, mg/l	0.46±0.20	0.38±0.23

Values are Mean ± SEM (n = 46 for NPJ and n = 54 for HPJ). #: Different from NPJ, P < 0.05. Apo; apolipoprotein; BMI, body mass index; BP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; HPJ: high-polyphenols orange juice; LDL-C, low-density lipoprotein cholesterol; NPJ: normal-polyphenols orange juice; SBP, systolic blood pressure; TAG, triacylglycerols; TC, total cholesterol; WC, waist circumference.

Dietary intake

Volunteers decreased protein and fat dietary intake during both interventions and increased CHO intake only during the NPJ intervention. For this reason, the total energy intake was significantly lower only when volunteers were having the HPJ. However, there were no significant differences in dietary intake (energy, macronutrients and fibre) between both interventions after 12-wk (**Table 8**).

Table 8. Dietary intake in overweight and obese adults, at baseline and after the 12-wk NPJ and HPJ interventions.

	NPJ		HPJ	
	Baseline	Final	Baseline	Final
Energy Intake, kcal/d	2141±61	2060±47	2254±62	2076±47*
Cholesterol intake, g/d	315±10	285±8*	318±9	285±9*
Fibre intake, g/d	24±1	23±1	24±1	24±1
Protein intake, g/d	102±3	94±2*	105±3	98±3*
CHO intake, g/d	220±7	241±6*	232±7	242±6
Fat intake, g/d	92±3	81±2*	97±3	79±2*
MUFA intake, g/d	44±1	38±1*	45±1	37±1*
PUFA intake, g/d	15±1	12±0*	16±1	13±1*
SFA intake, g/d	23±1	19±1*	24±1	19±1*
Water intake, g/d	1578±39	1824±39*	1628±40	1879±41*

Values are expressed as mean±SEM. (n = 100 each for NPJ and HPJ). * Differences from baseline, $P \leq 0.05$. No significant intervention effect or P-interactions were observed. CHO, carbohydrates; MUFA, monounsaturated fatty acids; HPJ, high-polyphenols orange juice; PUFA, polyunsaturated fatty acids; NPJ, normal-polyphenols orange juice; SFA, saturated fatty acids.

Before the intervention, weight, WC and BP (**Table 9**), serum glucose and lipid metabolism, plasma leptin (**Table 11**), urine flavanones conjugates **(Figure 25),** antioxidant and defence system biomarkers (**Table 13**) and plasma retinol (**Table 15**) and oxidative stress biomarkers **(Table 16)**, were similar in the two groups of intervention at baseline. In contrast, plasma concentrations of α-tocopherol, CoQ_9 and CoQ_{10} were significantly lower in the HPJ group ($P = 0.026$, $P = 0.003$ and $P \leq 0.001$, respectively), whereas β-carotene was significantly higher in the HPJ group ($P \leq 0.001$) compared with the NPJ group (**Table 15**).

Effect of orange juice intake on anthropometry

As shown on **Table 9**, weight, BMI, and WC decreased after the intake of both juices (all $P \leq 0.001$), while SBP and DBP decreased only after NPJ intervention ($P = 0.009$ and $P \leq 0.001$, respectively). Additionally, BMI and WC were correlated with energy intake, SBP, leptin, 8-OHdG, and 8-iso-PGF2α (**Table 10**). Nevertheless, there were no significant differences in the anthropometric parameters of the participants between the two interventions.

Table 9. Anthropometric characteristics in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions

	NPJ		HPJ	
	Baseline	Final	Baseline	Final
Weight, kg	90.4±1.5	89.1±1.5*	90.6±1.5	88.8±1.5*
BMI, kg/m^2	32.5±0.4	32.0±0.4*	32.6±0.4	31.9±0.4*
WC, cm	99.0±1.3	95.1±1.2*	99.4±1.1	95.6±1.1*
SBP, mm Hg	128±1	124±2*	127±1	124±1
DBP, mm Hg	79±1	76±1*	78±1	77±1

Values are expressed as Mean±SEM. (n = 100 each for NPJ and HPJ). *Differences from baseline, $P \leq 0.05$. No significant intervention effect or P-interactions were observed. BMI, body mass index; DBP, diastolic blood pressure, HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice; SBP, systolic blood pressure; WC, waist circumference.

Table 10. Spearman correlations between delta values of outcomes (baseline – post-intervention) BMI, WC and anthropometric and urinary oxidative stress biomarkers in all overweight and obese adults after 12-wk NPJ or HPJ interventions.

	BMI		WC	
	rho	p	rho	p
Energy intake	0.392	< 0.001	0.255	< 0.001
SBP	0.289	< 0.001	0.246	< 0.001
DBP	0.283	< 0.001	0.233	< 0.001
Leptin	0.266	< 0.001	0.312	< 0.001
8-OHdG	0.263	< 0.001	0.233	< 0.001
8-iso-PGF2α	0.223	< 0.001	0.225	< 0.001

Values presented correspond to Spearman´s (*rho*) correlations. The correlations were obtained using the delta (baseline – post-intervention) value of both groups pooled to increase the n and in order to evaluate the effect of doses of flavanones. When correlations were not significant, the *r* or *rho* and *P*- values are not given.8-OHdG, 8-hydroxydeoguanosine; 8-iso-PGF2α, isoprostanes; BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; WC, waist circumference. *rho* and p corresponds to a spearman rank correlation.

Effect of orange juice intake on serum glucose and lipid metabolism parameters and plasma leptin.

Data concerning to glucose and lipid metabolism are shown in **Table 11**. Serum glucose increased significantly after the intake of NPJ and HPJ ($P \leq 0.001$), while serum insulin concentrations decreased significantly after NPJ intake ($P = 0.04$) and tended to decrease after HPJ intake ($P = 0.06$). However, the juice effects on glucose, insulin and the HOMA-IR index were significantly higher after the HPJ intake than after the NPJ (glucose, $\beta = 0.017$ mmol/l, $P = 0.009$; insulin, $\beta = 1.31$ μU/ml, $P = 0.007$; the HOMA-IR index, $\beta = 0.38$, $P = 0.040$). Regarding lipid metabolism, TC, HDL-C and LDL-C serum concentrations were similar throughout the study with no significant differences between NPJ and HPJ interventions. Serum TAG and Apo-B concentrations decreased significantly only after the intake of the NPJ ($P = 0.049$ and $P \leq 0.001$, respectively), whereas ApoA-I significantly increased after the intake of HPJ ($P = 0.024$). Moreover, plasma leptin decreased after 12-wk intervention with either the NPJ or the HPJ ($P = 0.007$ and $P = 0.02$, respectively)

Table 11. Serum glucose and lipid metabolism parameters and plasma leptin in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions

	NPJ		HPJ		
	Baseline	Final	Baseline	Final	P
Glucose, mmol/l	4.9±0.1	5.2±0.1*	5.0±0.1	5.2±0.1*	**0.009**
Insulin, μU/ml	12.7±0.7	11.5±0.6*	13.8±0.9	12.7±0.7	**0.007**
HOMA-IR	2.86±0.21	2.70±0.16	3.13±0.25	3.04±0.19	**0.004**
TC, mmol/l	5.6±0.1	5.6±0.1	5.6±0.1	5.6±0.1	NS
HDL, mmol/l	1.29±0.03	1.32±0.03	1.32±0.03	1.29±0.03	NS
LDL, mmol/l	3.39±0.08	3.47±0.08	3.41±0.08	3.49±0.08	NS
TAG, mg/dl	132±6	124±6*	136±6	130±5	NS
ApoA-I, mg/dl	147±2	147±2	149±2	145±2*	NS
Apo-B, mg/dl	95±2	91±2*	96±2	93±2	NS
Leptin, ng/l	22.7±1.5	19.6±1.4*	22.9±1.9	20.6±1.6*	NS

Values are expressed as mean±SEM. (n = 100 each for NPJ and HPJ). * Differences from baseline, $P \leq 0.05$. Intervention effect: P-value indicates differences between the two interventions with LMM. $P \leq 0.05$ is considered significant. NS: non-significant effect, $P > 0.05$. Apo, apolipoprotein; HDL, high-density cholesterol; HPJ, High-polyphenols orange juice; HOMA-IR, homeostasis model assessment; LDL, low-density cholesterol; LMM, Linear mixed model; NPJ normal-polyphenols orange juice; TAG, triacylglycerides; TC, total cholesterol.

Serum TAG and Apo-B concentrations decreased significantly only after the intervention with the NPJ ($P = 0.049$ and $P \leq 0.001$, respectively), whereas ApoA-I increased significantly after the HPJ intake ($P = 0.024$). Moreover, plasma leptin decreased after 12-wk intervention with either the NPJ or the HPJ ($P = 0.007$ and $P = 0.020$, respectively).

Effect of orange juice intake on urinary polyphenols

The presence of urine flavanones was very low at baseline as shown in **Figure 25.** Urine hesperitin and naringenin metabolites increased after the intake of both OJs (all $P \leq 0.001$) but they were significantly higher after the HPJ intervention compared to the NPJ intervention ($P \leq 0.001$).

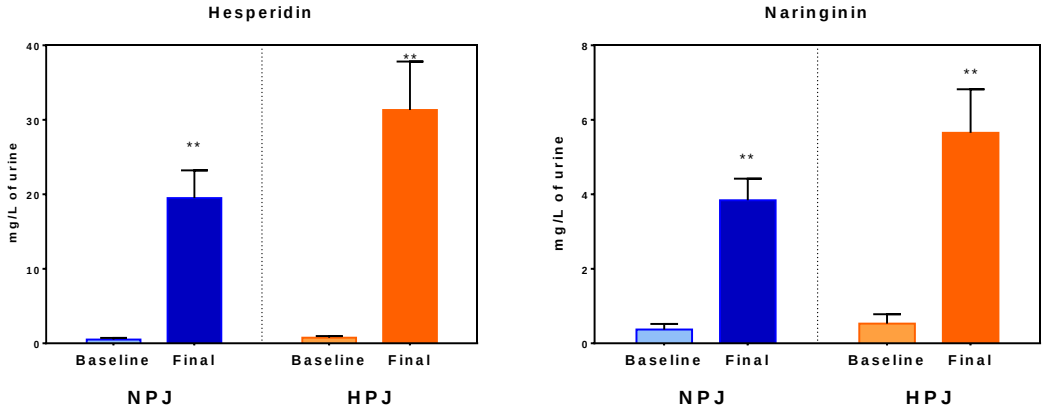

Figure 25. Urine hesperidin and naringin metabolites in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions. ** P ≤ 0.001 different from baseline. HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice

Moreover, when analysing the urinary concentration of both flavanones, we observed a significant interaction between the interventions and the arms of study (i.e., *P*-interaction; all ≤ 0.001) because the increase in flavanones was more pronounced during the first arm of the study than during the second arm (**Figure 26**).

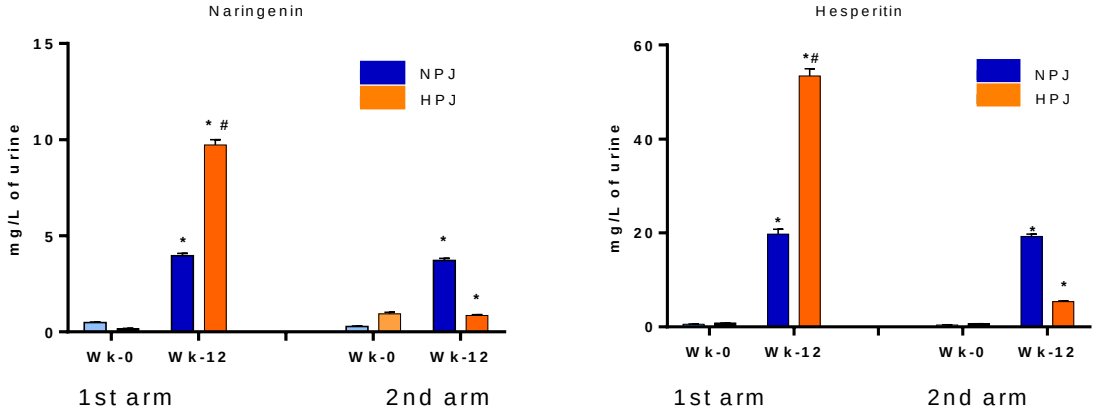

Figure 26. Urine concentration of hesperidin and naringenin conjugates of overweight and obese adults, at baseline and after 12-wk HPJ and NPJ interventions. Values are mean±SEM. n = 46 for NPJ and n = 54 for HPJ in the first arm and n = 54 for NPJ and n = 46 for HPJ during the second arm of the study. * Different from week 0, $P < 0.05$; in each arm of the study; # different from NPJ within each arm of the study, $P < 0.05$. HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice

Effect of orange juice intake on erythrocyte E-ADS activities

Table 12 summarizes the results related to the E-ADS. Intervention with either HPJ or NPJ induced a similar decrease in GR ($P \leq 0.001$ and $P = 0.031$, respectively) and catalase activities ($P \leq 0.001$, each). In contrast, SOD activity was significantly higher ($P = 0.008$) after HPJ consumption. However, the erythrocyte antioxidant enzyme activities and the related antioxidant molecules GSH and GSSH in plasma did not show any differences between both interventions. Additionally, no significant differences were observed in these enzymes by either interventions, neither *P*-interactions.

Table 12. Erythrocyte E-ADS in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions

	NPJ		HPJ	
	Baseline	Final	Baseline	Final
Catalase, nmol/(Lg Hb)	0.26±0.01	0.23±0.00*	0.26±0.01	0.22±0.01*
SOD, U/mg Hb	20.68±1.61	21.13±1.67	17.72±1.51	23.07±1.72*
GPX, U/g Hb	15.97±0.42	15.54±0.58	15.52±0.38	15.33±0.56
GR, U/g Hb	2.50±0.06	2.11±0.09*	2.33±0.06	2.18±0.07*
GSH, mg/l	5.8±0.7	6.3±0.8	7.6±0.8	6.1±0.8
GSSG, mg/l	42±3	44±3	40±3	46±3

Values are expressed as mean±SEM. (n = 100 each for NPJ and HPJ). * Differences from baseline, $P \leq 0.05$. No significant intervention effect or *P*-interactions were observed. GPX, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase.

Erythrocyte catalase activity was inversely correlated with hesperetin, naringenin and GR, whereas it was positively correlated with 8-OHdG and 8-iso-PGF2α. In contrast, SOD activity was positively correlated with GPX and GR. (**Table 13**).

Table 13. Spearman correlations between erythrocyte delta (baseline – post-intervention) E-ADS, urinary polyphenols and oxidative stress biomarkers in all overweight and obese at baseline and after 12-wk NPJ and HPJ interventions

	Catalase		SOD	
	rho	*P*	*rho*	*P*
Hesperidin	-0.17	0.013	-	-
Naringenin	-0.19	0.005	-	-
GR	-0.15	0.015	0.15	0.020
GPX	-	-	0.29	≤ 0.001
8-OHdG	0.29	≤ 0.001	-	-
8-iso-PGF$_{2\alpha}$	0.25	≤ 0.001	-	-

Values presented correspond to Spearman´s (rho) correlations. The correlations were obtained using the delta (baseline – post-intervention) value of both groups pooled to increase the n and in order to evaluate the effect of doses of flavanones. When correlations were not significant, the r or rho and P-values are not given. 8-iso-PGF2α, isoprostanes; 8-OHdG, 8'dehydroxyguanosine; GPX, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase.

Effect of orange juice intake on plasma NEADS parameters

Results related to NEADS are shown in **Table 14**. At baseline, CoQ9 and CoQ$_{10}$ plasma concentrations were lower and β-carotene concentration was higher after HPJ intervention

compared to NPJ intervention (all $P \leq 0.05$). Initial and final data showed an increase in CoQ_{10} ($P = 0.019$) after HPJ intake, whereas an increase in β–carotene was observed after NPJ intake ($P = 0.001$). After adjusting for age and sex, we observed higher plasma concentrations of retinol ($P = 0.009$), α-tocopherol ($P = 0.004$), β–carotene ($P = 0.008$) and CoQ_{10} ($P = 0.001$) in older subjects; male subjects had higher concentrations of retinol ($P \leq 0.001$) and CoQ_{10} ($P \leq 0.001$). There was a significant effect between the groups for plasma CoQ_9 ($P = 0.002$) but not for plasma retinol, α-tocopherol, β–carotene and CoQ_{10}. Additionally, increased BMI was associated with lower plasma β-carotene concentration. The concentrations of these variables, with the exception of CoQ_9 ($P = 0.008$), were similar at the end of the NPJ and HPJ interventions. Finally, CoQ_9 was correlated with CoQ10 ($r = 0.63$ $p \leq 0.001$).

Table 14. Plasma NEADS parameters in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions

	NPJ		HPJ		
	Baseline	Final	Baseline	Final	P
Retinol, mg/ml	0.24±0.01	0.27±0.01*	0.24±0.01	0.26±0.01	NS
α-Tocopherol, mg/ml	11.1±0.2	11.6±0.2	11.1±0.3	12.1±0.2*	NS
β-Carotene, mg/ml	0.39±0.03	0.68±0.05*	0.65±0.04$^{\neq}$	0.63±0.04	NS
CoQ_9, mg/ml	7.34±0.23	4.73±0.38*	5.54±0.26$^{\neq}$	6.07±0.23	0.002
CoQ_{10}, mg/ml	338.1±9.6	295.9±11.2*	265.9±10.0$^{\neq}$	308.9±9.6*	NS

Values are expressed as mean±SEM. (n = 100 each for NPJ and HPJ). * Differences from baseline, $P \leq 0.05$. Intervention effect: P-value indicates differences between the two interventions with LMM. $P \leq 0.05$ is considered significant. NS: non-significant effect, $P > 0.05$. CoQ, Coenzyme; HPJ, LMM, linear mixed model, HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

Effect of orange juice intake on oxidative stress biomarkers

Regarding oxidative stress biomarkers, data is shown in **Table 15**. Urinary 8-OHdG and 8-iso-PGF2α decreased after the 12-wk NPJ and HPJ interventions (all $P \leq 0.001$).

Additionally, urinary 8-OHdG was lower after HPJ intake compared to NPJ intake ($P = 0.012$). Indeed, there was a significant *P*-interaction between the interventions and the arms of the study (*P*-interaction = 0.002), since during the first arm of the study, NPJ intake caused a greater decrease in urinary 8-OHdG than HPJ intake ($P = 0.01$), whereas in the second arm of the study, NPJ intake induced an increase while HPJ induced a decrease in this parameter ($P =$

0.012) (**Figure 27**). There was no significant difference in 8-iso-PGF2α concentrations between the two interventions. Additionally, we observed a positive correlation between 8-OHdG and 8-iso-PGF2α and negative correlations between 8-OHdG, CoQ_9 and CoQ_{10} and between 8-iso-PGF2α and β-carotene (**Table 16**). After adjusting for age, we observed that urine 8-iso-PGF2α concentration was higher in younger subjects and diminished with increasing age. On the contrary, plasma LPO decreased following the NPJ intervention ($P = 0.002$) but was maintained after the HPJ intervention. A significant difference in LPO was observed between the two interventions ($P = 0.003$), and the LPO values were lower in males than in females ($P \leq 0.001$). Moreover, LPO was inversely correlated with α-tocopherol.

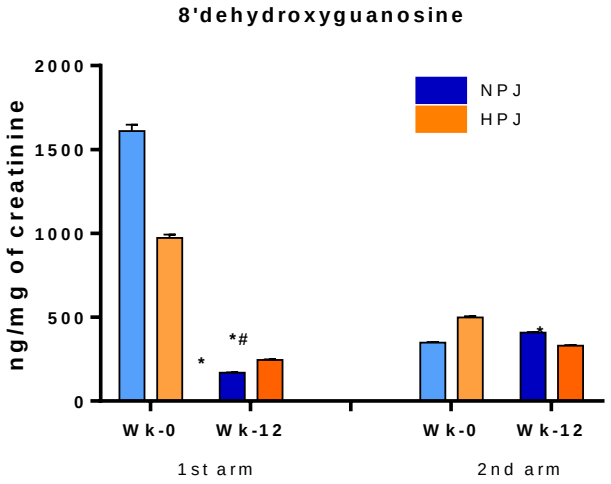

Figure 27. Urine concentration of 8-OHdG in overweight and obese adults, at baseline and after 12-wk HPJ and NPJ interventions. Values are mean±SEM. n = 46 for NPJ and n = 54 for HPJ in the first arm and n = 54 for NPJ and n = 46 for HPJ during the second arm of the study. * Different from week 0, P < 0.05; in each arm of the study; # different from NPJ within each arm of the study, P < 0.05. 8-OHdG, 8-hydroxy-2'-deoxyguanosine; HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice

Table 15. Oxidative stress biomarkers in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions

	NPJ		HPJ			
	Baseline	Final	Baseline	Final	*P*	*P*-interaction
Urine 8-iso-PGF2α, ng/mg creatinine	436±69	155.6±14.2*	347±44	154±13*	NS	NS
Urine 8-OHdG, ng/mg creatinine	934±134	298±20*	749±84	285±17*	**0.01**	**0.002**
Plasma LPO, µmol/l	0.75±0.03	0.69±0.03*	0.75±0.03	0.77±0.03	**0.01**	**0.050**
Plasma MDA, µmol /l	22.1±1.4	20.8±1.3	19.8±1.2	20.3±1.2	NS	NS
Plasma OxLDL, ng/l	334±41	343±38	322±39	326±40	NS	NS

Values are expressed as mean±SEM. (n = 100 each for NPJ and HPJ). * Differences from baseline, P ≤ 0.05. Intervention effect: P-value indicates differences between the two interventions with LMM. P ≤ 0.05 is considered significant. NS: non-significant effect, P > 0.05. 8-iso-PGF2α, isoprostanes; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; OxLDL: Oxidised LDL; HPJ, high-polyphenols orange juice; LMM, linear mixed model; LPO, lipid peroxides; MDA, malonaldehyde. NPJ, normal-polyphenols orange juice

Table 16. Spearman correlations between delta values (baseline – post-intervention) for oxidative stress biomarkers, urinary hesperidin and plasma NEADS parameters in all overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions

	β-carotene		LPO		MDA		8-OHdG		CoQ_9	
	rho	*P*	*rho*	*P*	*rho*	*P*	*rho*	*P*	*rho*	*P*
Hesperidin	-	-	-	-	0.16	0.013	-	-	-	-
CoQ_9	-	-	-	-	-	-	-0.17	0.016	-	-
CoQ_{10}	-	-	-	-	-	-	-0.17	0.023	0.63	≤ 0.001
α-tocopherol	-0.15	0.05	-0.17	0.016	-	-	-	-	-	-
8-iso-$PGF_{2\alpha}$		-	-	-	-	-	0.72	≤ 0.001	-	-

Values presented correspond to Pearson's (r) or Spearman´s (rho) correlations. The correlations were obtained using the delta (baseline – post-intervention) value of both groups pooled to increase the n and in order to evaluate the effect of doses of flavanones. When correlations were not significant, the r or rho and P-values are not given. 8-iso-PGF2α, isoprostanes; 8-OHdG, 8'dehydroxyguanosine; CoQ, coenzyme Q; LPO, lipid peroxidation; MDA, Malondialdehyde

.

Finally, there were no juice effects on plasma MDA and oxLDL concentrations, and the concentrations did not significantly differ between the two interventions; however, we found that plasma MDA was lower in females ($P \leq 0.001$). Additionally, we observed a relationship between hesperitin and MDA (**Table 16**).

Effect of orange juice intake on inflammatory biomarkers

We found that the 12-wk intervention with the NPJ increased plasma IL-1 and IL-6 (**Table 17**). On the other hand, the 12-wk intervention with the HPJ decreased CRP, IL-6 and tPAI-1 (**Table 17**). These effects on IL-1 and IL-6 were significantly different after both interventions ($P \leq 0.017$ and $P \leq 0.001$, respectively). Nonetheless, there was a *P*-interaction on IL-1, IL-8 and TNF-α ($P \leq 0.001$), due to a significant better response to the intervention with the HPJ in the first arm of the study and a non-significant response during the second arm of the study.

Table 17. Inflammatory biomarkers in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions.

	NPJ		HPJ			
	Baseline	Final	Baseline	Final	*P*	*P*-interaction
CRP, mg/l	0.72±0.16	0.86±0.17	1.20±0.18	0.32±0.08*	NS	NS
IL-1, ng/l	0.70±0.03	0.53±0.03*	0.49±0.03	0.47±0.03	**0.017**	**<0.001**
IL-6, ng/l	2.83±0.15	3.58±0.14*	3.45±0.13	3.09±0.13*	**<0.001**	NS
IL-8, ng/l	1.42±0.08	1.57±0.07	1.42±0.06	1.30±0.07*	NS	**<0.001**
TNF-α, pg/ml	31.2±1.2	32.9±0.9	31.1±0.9	28.6±1.0*	NS	**<0.001**
tPAI-1, µg/l	52.4±3.2	46.5±2.5	61.2±3.6	51.8±3.0*	NS	NS

Values are expressed as Mean±SEM. (n = 100 each for NPJ and HPJ). * Differences from baseline, $P \leq 0.05$. Intervention effect: P-value indicates differences between the two interventions with LMM. $P \leq 0.05$ is considered significant. NS: non-significant effect, $P > 0.05$. CRP, C-reactive protein; IL, interleukin; LMM, linear mixed model; TNF, tumour necrosis factor; PAI, plasminogen activator inhibitor.

Metabolomics approach to the identification of biomarkers of orange juice intake, inflammation and oxidative stress

General characteristics of subjects

Table 18 shows the anthropometric characteristics, body composition and serum biochemical parameters before the NPJ and HPJ interventions in the subsample of 30 subjects included in the metabolomics analysis. There were no significant differences between groups in anthropometry or biochemical parameters at baseline.

Table 18. Anthropometric characteristics, body composition and serum biochemical parameters in overweight and obese adults, before the HPJ and NPJ interventions.

	NPJ	HPJ	T-student
n	15	15	
Age, y	46±2	42±3	NS
SBP, mm Hg	127±4	131±4	NS
DBP, mm Hg	81±3	80±2	NS
BMI, kg/m^2	32.3±1.1	32.0±0.9	NS
Weight, kg	89.3±3.4	91.4±4.2	NS
Body Fat, %	32.8±2.4	33.3±2.6	NS
WC, cm	100±3	99±3	NS
Glucose, mg/dL	85±2	83±3	NS
Insulin, µU/ml	13.3±1.2	15.3±2.6	NS
HOMA, mg/dL	2.8±0.3	3.3±0.7	NS
CT, mg/dL	201±8	203±9	NS
HDL, mg/dL	51±3	55±5	NS
LDL, mg/dL	119±6	120±7	NS
TAG, mg/dL	140±17	108±8	NS

Values are expressed as Mean±SEM. T-student correspond to comparison between groups. NS: non-significant effect, $P > 0.05$. BMI, body mass index; CT, total cholesterol; DBP, diastolic blood pressure; HDL-c, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; LDL-c, low-density lipoprotein cholesterol; HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice; SBP, systolic blood pressure; TAG, triacylglycerols; WC, waist circumference.

Metabolites detection and univariate analysis

Plasma metabolic profiling was established to explore important biomarkers and metabolic patterns related to the OJ consumption. Six hundred fifty-one metabolites of known identity were detected using the Metabolon's platform, 33 corresponding to the GC-MS platform, 321

corresponding to the LC/MS positive mode, 221 corresponding to the LC/MS negative mode and 76 corresponding to the LC/MS polar mode.

Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, ANOVA contrasts were used to identify metabolites that differed significantly between baseline and after the intervention with both OJs and within the two interventional groups. Sixty-one metabolites were different between the HPJ and NPJ samples at baseline, however, after the intervention, only 23 differed. Meanwhile, when metabolites were compared across the time, there was a shift of 100 metabolites in the NPJ intervention and 131 after the HPJ intervention. A summary of the numbers of metabolites that achieved statistical significance ($P \leq 0.05$) when comparing baseline *vs* final samples and NPJ *vs* HPJ is shown in the **Table 19**.

Table 19. Summary of the number of significant metabolites after ANOVA contrasts and repeated measures ANOVA that differs between baseline *vs* post NPJ and post-HPJ interventions, and between both groups at baseline and at the end of both 12-wk interventions in overweight and obese adults included in metabolomics study

ANOVA Contrasts	**HPJ NPJ**		**Final Baseline**	
	Baseline	**Final**	**NPJ**	**HPJ**
Total biochemicals $p \leq 0.05$	61	23	100	131
Biochemicals (↑↓)	53|8	16|7	63|37	52|79
Total biochemicals $0.05 < p < 0.10$	38	33	54	41
Biochemicals (↑↓)	30|8	17|16	34|20	12|29

Repeated Measures ANOVA	**Intervention**	**Time**	**Intervention:Time**
	Main Effect	**Main Effect**	**Interaction**
Total biochemicals $p \leq 0.05$	38	156	49

ANOVA, analysis of variance, HPJ, high-polyphenols orange juice, NPJ, normal-polyphenols orange juice. ↑ increase ↓ decrease

Multivariate analysis: the use of principal component analysis for the identification of metabolite patterns.

The explorative unsupervised multivariate analysis method PCA was used for the detection of trends, patterns and clustering among samples (subjects) and variables (possible biomarkers). The clustering data failed to differentiate baselines and NPJ or HPJ post-intervention samples, as it is shown in **Figure 28**. Similarly, no clusters were observed separating both interventions.

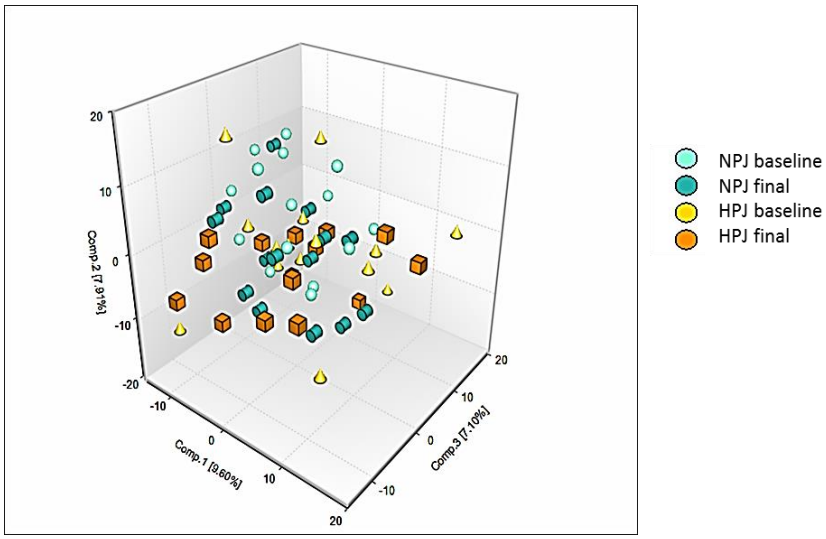

Figure 28. PCA loadings representing data from overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions showed that, there was no clustering among samples. HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice; PCA: principal component analysis.

Figure 29 shows the influence of gender in metabolome. Clusters differentiating both genders are observed in serum metabolome regardless dietary status, further highlighting the limited number of biochemical differences induced by OJ consumption.

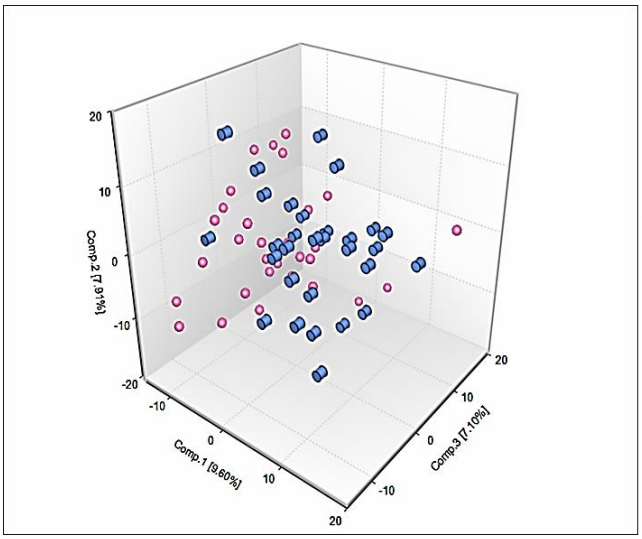

Figure 29. PCA loadings representing data from male and female overweight and obese adults, showed a clear separation between both genders. PCA, principal component analysis.

Random forest analysis for the identification of discriminating patterns

RF analysis is a supervised classification technique. It is based on an ensemble of decision trees and has been proven a reliable tool for identifying biomarkers. Remarkably, an unique different biochemical signature was observed between HPJ baseline and post-HPJ samples. The top 30 metabolites included in this signature are detailed in **Figure 30**, where the metabolites in the X-axis are ordered in an increased order, according to its importance for the assignation to the pre- or post-HPJ groups, while the Y-axis gives the MDAC value. Interestingly, three of the top five metabolites, with a MDAC>40, are those related to OJ consumption, i.e. methyl glucopyranoside (alpha-beta), stachydrine and betonicine. When analysing HPJ samples, the predictive accuracy is 97%, indicating that for the 15 samples analysed, 14 could be classified correctly when using this biochemical signature.

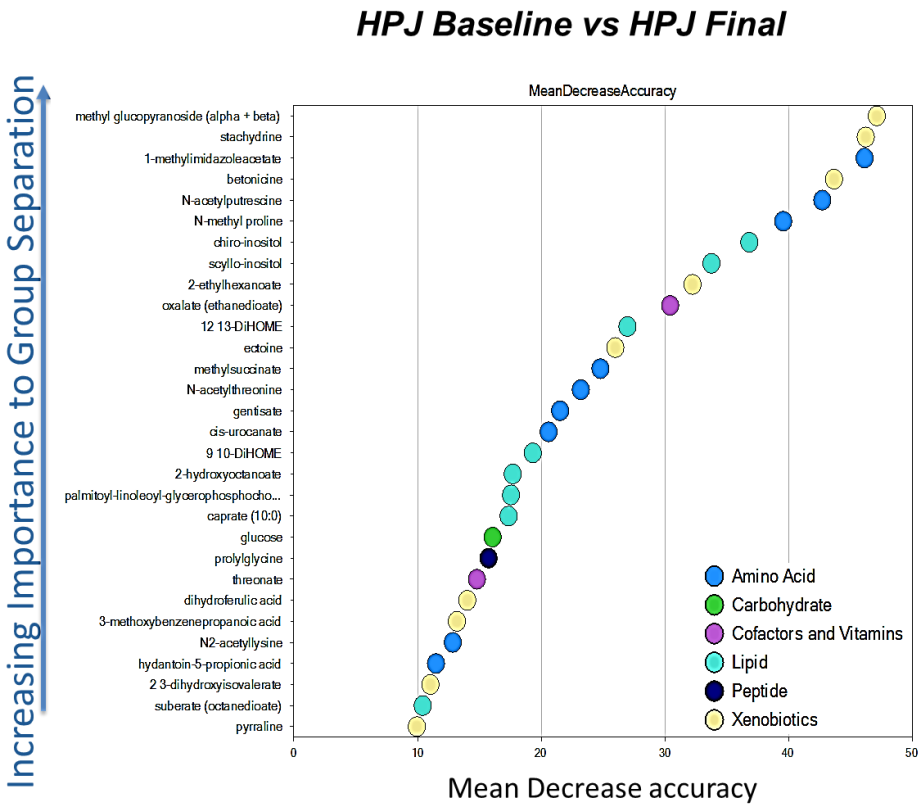

Figure 30. Random forest analysis of plasma metabolome in overweight and obese adults, at baseline and after 12-wk HPJ intervention. HPJ, high-polyphenols orange juice.

On the other hand, RF analysis comparing baseline and post NPJ intervention samples indicates that those metabolites with a higher contribution to the metabolic signature found in the HPJ are stachydrine, and N-methyl proline and betonicine, in this case with a MDAC > 30 (**Figure 31**) and a predictive accuracy of 64%. This indicates that only 9 of the 15 samples could be classified correctly based on this metabolic signature. This is unacceptable since the random chance will influence the classification of a sample more than the NPJ intervention.

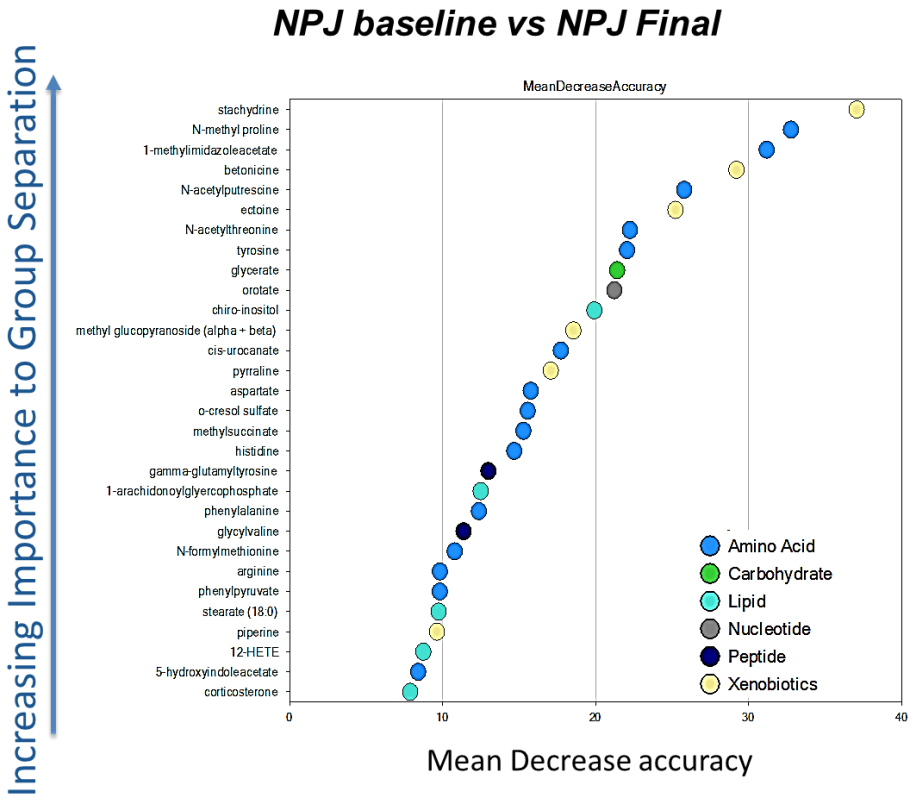

Figure 31. Random forest analysis of plasma metabolome in overweight and obese adults, at baseline and after 12-wk NPJ intervention. NPJ, normal-polyphenols orange juice.

Finally, the analysis comparing NPJ and HPJ samples shows that 12,13-dihydroxy-octadecenoic acids (12,13-DiHOME) is the most important metabolite for discriminate between groups. At baseline, the MDAC = 30 and the predictive accuracy of 70% allowing discrimination between groups; however, the capacity to use 12,13-DiHOME to discriminate between samples after the interventions with NPJ and HPJ had a MDAC < 0 and a predictive accuracy of 56%, suggesting low capacity to discriminate between post NPJ and HPJ samples.

Identification of biomarkers related to orange juice consumption

Potential evidence of the biochemical signature of OJ consumption identified by RF analysis was confirmed by LC-MS/MS. **Figures 32** to **37** represents changes on different biomarkers associated to the OJ consumption before and after the NPJ and HPJ interventions. The vertical axis represents the median scaled intensity for each metabolite.

Biomarkers related with orange juice intake

Intervention with both OJs increased serum levels of stachydrine (NPJ, FC = 2.49; HPJ, FC = 3.89; *time effect* q < 0.0001), methyl glucopyranoside (NPJ, FC = 2.13; HPJ, FC = 6.52; *time effect* q < 0.0001), and betonicine (NPJ, FC = 2.07; HPJ, FC = 11.01; *time effect* q < 0.0001) (**Figure 32a**). In addition, galactonate increased in both after the 12-wk interventions with either NPJ or HPJ (NPJ, FC = 1.71; HPJ, FC = 1.40; *time effect* q = 0.0024) (**Figure 32b**). However, there were no significant effect of the intervention nor intervention per time interaction.

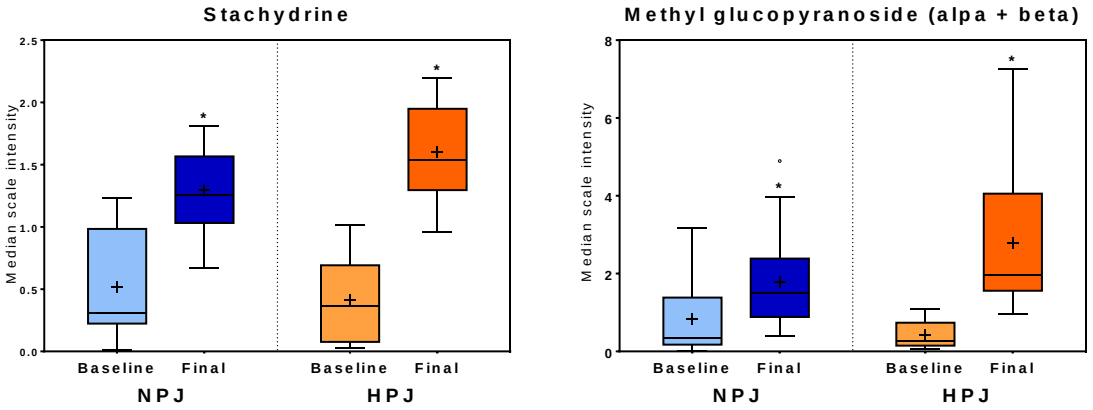

Figure 32a. Serum levels of stachydrine, methyl glucopyranoside, in overweight and obese adults, at baseline, and after 12-wk NPJ and HPJ interventions. ○, outlier; ** different from baseline (q < 0.01), * different from baseline (q < 0.1). HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

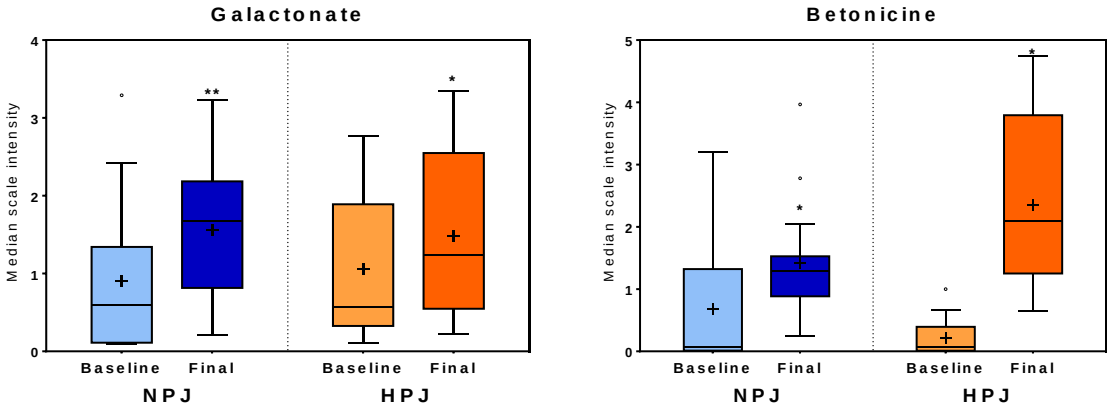

Figure 32b. Serum levels of betonicine and galactonate in overweight and obese adults, at baseline, and after 12-wk NPJ and HPJ interventions. ○, outlier; ** different from baseline (q < 0.01), * different from baseline (q < 0.1). HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

Figure 33 shows differences in the levels of metabolites related to polyphenol intake, dihydroferulic acid and ferulic acid 4-sulphate, in samples from overweight and obese adults that took NPJ or HPJ during 12 wk. Dihydroferulic acid was accumulated after the intervention with the HPJ compared to baseline (FC = 5.00; *time effect* q = 0.047) but not after the 12-wk intervention with the NPJ. Serum levels of dihydroferulic acid tended to be higher in the HPJ compared to LPJ (p = 0.015), however, the q-value was above the cut-off for FDR (FC = 4.91; *intervention x time* q = 0.2774). Similarly, ferulic acid 4-sulphate was also accumulated after the 12-wk intervention with the HPJ (FC = 1.80, time effect q = 0.1) but not with the NPJ (**Figure 33**). Other biomarkers related to OJ-associated polyphenols, such as hesperetin and its glycosides, were below the limit of detection. On the other hand, ferulic acid 4-sulphate was correlated with urinary hesperidin (rho = 0.382; *P* =0.037) and naringin (rho = 0.409; *P* =0.025).

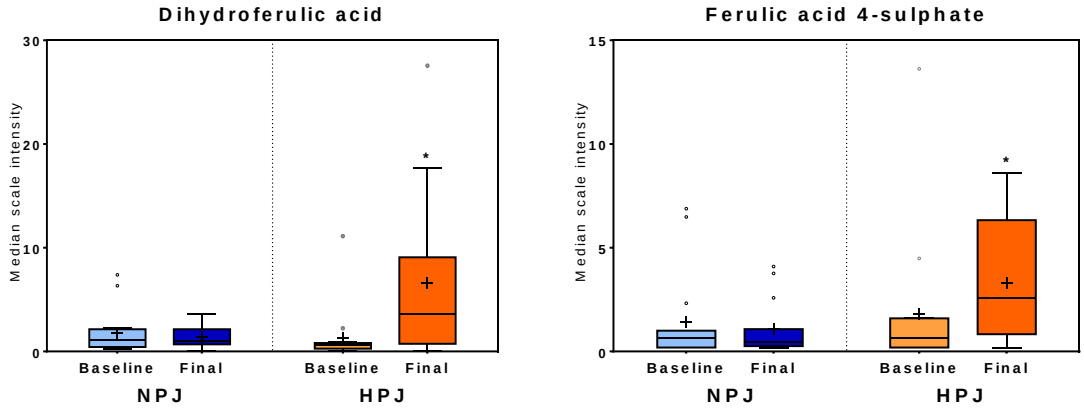

Figure 33. Serum levels of dihydroferulic acid and ferulic acid 4-sulphate in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions. ○, outlier. *, different from baseline (q < 0.01). HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

Figure 34 shows differences in the levels of metabolites related to vitamin C (ascorbate) intake, threonate and oxalate, in samples from overweight and obese adults that took NPJ or HPJ during 12 wk. After the intervention with both NPJ and HPJ, we observed increased serum levels of the ascorbate-derived product, threonate (NPJ, FC = 1.28; HPJ, FC =1.29; *time effect* = 0.006, q = 0.033) and oxalate (NPJ, FC = 1.19; HPJ, FC = 1.28; *time effect* = 0.018, q = 0.015).

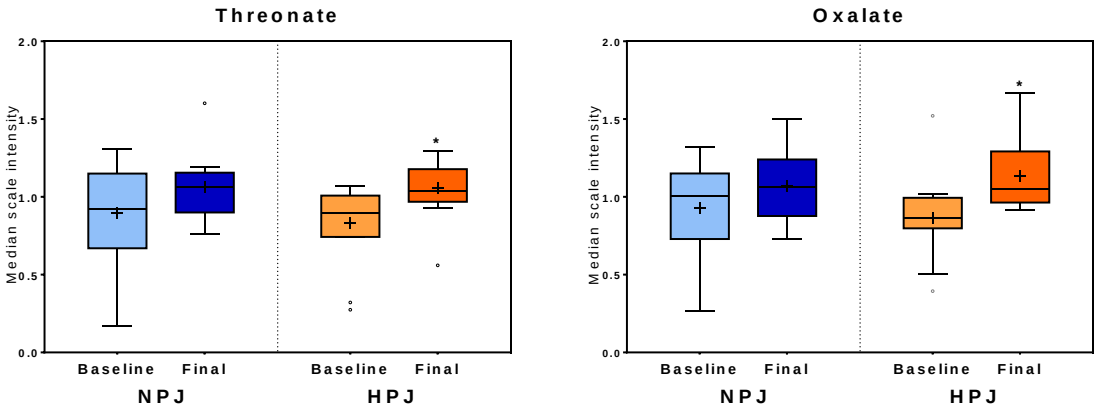

Figure 34. Serum levels of oxalate and threonate in overweight and obese, at baseline and after 12-wk NPJ and HPJ interventions. ○, outlier. * different from baseline (q < 0.1). HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

Metabolites related to oxidative stress and inflammation

Figure 35 shows differences in the levels of metabolites related to lipoxygenation products derived from linoleic acid, 9-Hydroxy-10,12-octadecadienoic (9-HODE) acid plus 13-hydroxy-9,11-octadecadienoic acid (13-HODE), in samples from overweight and obese adults that took NPJ or HPJ during 12 wk. serum levels of 9-HODE + 13-HODE were significantly diminished only after the 12-wk intervention with the HPJ (FC: 0.50; q = 0.0421) but not after the NPJ intervention. When analysing correlations, these metabolites were correlated with 9,12-dihydroxy-octadecenoic acids (9,12-DiHOME) and with the 12,13-DiHOME and inversely correlated with betonicine and naringin (**Table 20**)

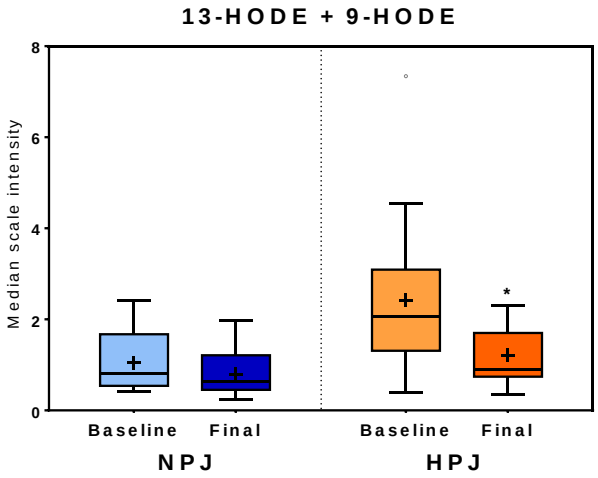

Figure 35. Serum levels of 13-HODE + 9-HODE in overweight and obese, at baseline and after 12-wk NPJ and HPJ interventions. ○, outlier. * different from baseline (q = 0.04). HODE; hydroxyoctadecadienoic acid; HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice

Table 20. Spearman correlations between oxidative stress and orange juice related metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions.

	9 + 13-HODE	
	rho	p
9,12-DiHOME	0.401	0.028
12,13-DiHOME	0.449	0.013
Betonicine	-0.399	0.029
Naringin	-0.428	0.018

DiHOME, dihydroxy-octadecenoic acids HODE, hydroxy-octadecadienoic acid.

Serum levels of the arachidonic-derived eicosanoid 5- hydroxyeicosatetraenoic acid (5-HETE) increased after the 12-wk NPJ intervention compared to baseline (FC = 1.48; $q < 0.1$) but not after the HPJ intervention, while serum 12-HETE increased after the HPJ intervention compared to baseline (FC = 5.27; $q < 0.06$) but not after the NPJ intervention (**Figure 36**).

Finally, the derivate dihydroxy fatty acids 12,13-DiHOME and 9,10- diminished only after the 12-wk intervention with the HPJ (FC = 0.41 and 0.51, respectively, q < 0.01) (**Figure 37**).

Both, 12,13-DiHOME and 9,10-DiHOME were inverse correlated with ferulic acid 4-sulphate (*rho* = -0.393, p = 0.032 and *rho* = -0.393, p = 0.011, respectively).

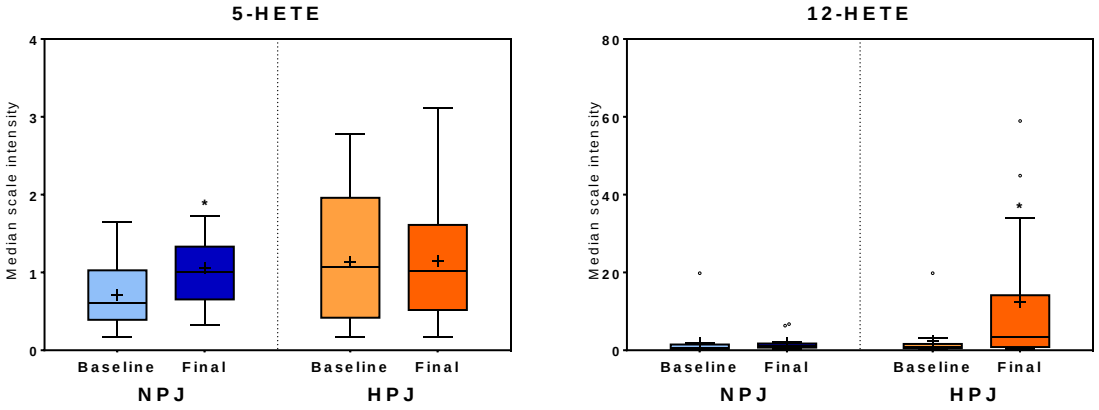

Figure 36. Serum levels of 5-HETE and 12-HETE in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions. ○, outlier.* different from baseline (q < 0.1). HETE, Hydroxyeicosatetraenoic acid; HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

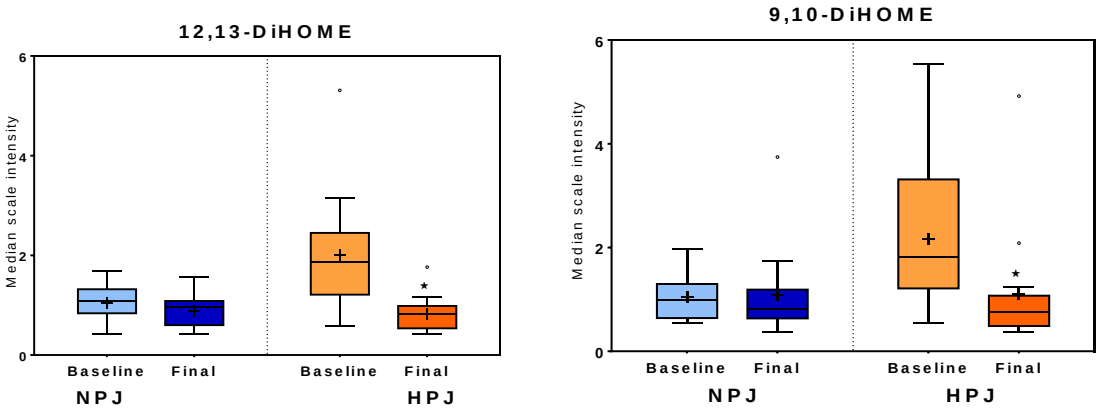

Figure 37. Serum levels of 12,13-DiHOME and 9,10-DiHOME in overweight and obese adults, at baseline and after 12-wk NPJ and HPJ interventions. ○, outlier; * different from baseline (q < 0.01) DiHOME, dihydroxy-octadecenoic acid; HPJ, high-polyphenols orange juice; NPJ, normal-polyphenols orange juice.

DISCUSSION

DISCUSSION

General findings

The present study demonstrates that the 12-week intervention with OJ, regardless of its polyphenols content (299 or 745 mg/d), improves the antioxidant defence system and thus may reduce initial lipid peroxidation products (i.e., plasma LPO), and advanced oxidation products (i.e., 8-iso-PGF2α and 8-OHdG). These findings indicate lower lipid and DNA oxidative damage after the interventions in non-smoking subjects who were either overweight or obese. Urinalysis and metabolomics analysis in serum demonstrated the presence of flavanone derivatives and metabolites derived from OJ compounds, which confirmed the subjects' adherence to the intervention and the bioavailability of the flavanones contained in the products. The metabolomics approach also shown new specific metabolites related to OJ and polyphenols consumption and supported other metabolites such as proline betaine (stachydrine) and ferulic acid already reported (Pujos-Guillot *et al.* 2013). In addition, we have found new metabolites related to the improvement oxidative stress and inflammation using metabolomics.

Notwithstanding, the 12-week intervention with OJ did not affect the anthropometric parameters and carbohydrate and lipid metabolism biomarkers. Furthermore, we did not find significant changes on NEADS, PAI-1 nor inflammatory biomarkers after juice consumption. Besides, the metabolomics PCA analysis failed to clusters data within interventions, showing similar metabolic patterns.

Orange juice and flavanones intake

Despite, we did not find tracks of hesperidin and narirutin and its glycosides in serum; we found that the concentration of their metabolites in urine, interestingly it was found double fold after intake of HPJ compared with the NPJ. Pujos-Guillot *et al.* (2013) reported a weak but significant correlation between citrus intake and urinary recovery of flavanones. A recent work found that urine excretion of hesperitin metabolites is around 17.5% of the total intake, while urine naringin metabolites excretion is around 12.7% of the ingested naringenin (Pereira-Caro *et al.* 2014). On the other hand, dihydroferulic acid and ferulic acid 4-sulphate have been described as metabolites associated to polyphenols consumption (Pereira-Caro *et al.* 2014; Llorach *et al.*

2014). Heinzmann *et al.* (2010) and Lloyd *et al.* (2010), have established stachydrine, and possible some of its metabolites, as valid biomarkers of citrus consumption. Additionally, Llorach *et al.* (2014) identified the same compound plus ferulic acid as biomarkers of a beverage containing 95% of citrus. In agreement with this, and regardless of predictive accuracy, RF analysis identified candidate biomarkers for differentiating pre- and post-dietary intervention data that may reflect OJ consumption and polyphenol intake according to previous evidences (Heinzmann *et al.* 2010; Lloyd *et al.* 2011; Pujos-Guillot *et al.* 2013). The increased levels of betonicine, stachydrine and methyl glucopyranoside after intervention of both juices and the presence of these metabolites in the top for discriminate between initial and post-intervention, with both NPJ and HPJ made them possible OJ consumption biomarkers. Additionally, we decided to include galactonate, regardless it was not in the top metabolites, due to the potential role derived from its origin from pectin present in orange peel, (Thakur, Singh, and Handa 1997). Putting together these metabolites must be possible to design an index instead of only one biomarker that may increase the specificity and avoid the bias derived from the individual inter-variability.

Interestingly, we have found increased serum levels of ferulic acid 4-sulphate and dihydroferulic acid after both interventions. Moreover, the concentration of ferulic acid 4-sulphate was associated with flavanones found in urine. This may be related to the proposed flavanones metabolism and mediated by colonic microbiota (Del Rio *et al.* 2010); however, the other proposed metabolite, dihydroferulic acid, was not correlated with urine flavanones. On the other hand, using the metabolomics approach we have observed that ascorbate-related metabolites, threonate and oxalate, were accumulated after the intervention in both NPJ and HPJ, confirming the increased intake of ascorbate associated to these interventions. All these data confirm the adherence of volunteers to the OJ interventions. The presence of diverse metabolites related to the gut microbiota derived from the orange juice consumption shows the importance of the interaction between diet and the gut: indeed, further analysis i.e. stool metabolomics, should be taken into account.

Orange juice and biomarkers of oxidative stress and the antioxidant defence system

To our best knowledge, this is the first long-term and large-scale crossover intervention trial to evaluate the effects of citrus juices with different doses of flavanones (mainly hesperidin and

narirutin conjugates), on the antioxidant defence system, oxidative stress biomarkers and serum metabolome in overweight and obese adults with clinical characteristics of MS.

There is limited evidence confirming that polyphenol-rich products are able to decrease lipid peroxidation (Hollman *et al.* 2011). Our data show that the intervention with both juices reduced urinary 8-iso-PGF2α, which is recognized as one of the most reliable indices of lipid peroxidation because of its specificity and stability. These data agree with Sanchez Moreno *et al.* (2003) who reported that drinking OJ increased vitamin C concentrations and reduced oxidative stress *in vivo* by lowering the concentration of 8-iso-PGF2α. However, we did not find a consistent effect on lipid peroxidation biomarkers in plasma. In fact, neither oxLDL nor MDA were modified, and LPO was only reduced after consumption of flavanones at 300 mg/d but not at 745 mg/d. To our best knowledge, no human dietary interventional studies using these flavanones and focusing on these biomarkers have been previously reported. Interestingly, data provided by the metabolomics analysis showed that 9-HODE plus 13-HODE, which are components derived from lipid peroxidation in the atherosclerotic plaque and are considered as biomarkers of oxidative stress (Vangaveti, Baune, and Kennedy 2010), were diminished after the intervention in the HPJ group. Furthermore, these metabolites were correlated with SBP suggesting a possible relation between de decrease of lipid peroxidation and BP improvement. By contrast, urinary naringinin and serum betonicine were inversely correlated with 9-HODE plus 13-HODE, confirming the antioxidant properties of the flavanones and other OJ compounds. Contrarily to these results, another study observed a dose-dependent pro-oxidant effects of naringenin and hesperidin (Yen *et al.* 2003), whereas Galati *et al.* (2004) have identified naringenin, but not hesperidin, to induce lipid peroxidation under the same conditions in which other flavanones exert antioxidant effects. Moreover, a recent review has reported the pro-oxidant effects of high doses of flavonoids (Carocho and Ferreira 2013). Therefore, more studies are needed in order to reach a conclusion about the doses of flavanones exerting antioxidant or pro-oxidant effect.

Several studies have established that supplementation with antioxidants appears to protect against DNA damage (Wu *et al.* 2004; Tirkey *et al.* 2005; Kawashima *et al.* 2007). As expected, we found that intervention with either of the experimental beverages produced a significant decrease in urinary 8-OHdG concentrations. Moreover, the observations that 8-OHdG was negatively correlated with CoQ_9 and CoQ_{10}, and that 8-iso-PGF2α was negatively correlated with β-carotene and CoQ_9, reflecting the importance of vitamin balance for the decrease of oxidative

DNA damage. In agreement with our results, it is well accepted that the intake of exogenous antioxidants modifies the NEADS in plasma parameters (Jomova and Valko 2013; Morand *et al.* 2011) and specifically, that the intake of OJ, hesperidin or narirutin may impact the NEADS (Landete 2013). Naringenin maintains a vitamin E sparing effect, which can lead to the neutralization of unsaturated membrane lipid peroxidation through its oxygen-scavenging effects (Mahmoud *et al.* 2012; Niki 2014). Moreover, hesperidin antioxidant activity, which mostly involves scavenging hydroxyl radicals and superoxide, is more efficient when hesperidin is combined with vitamin C, a compound that is naturally present in OJ (Choi 2008; Codoñer-Franch *et al.* 2008; Garg *et al.* 2001; Wilmsen, Spada, and Salvador 2005).

Our data demonstrated differences in baseline plasma antioxidant molecules; however, after the interventions, the plasma NEADS parameters improved independently of the type of OJ intake, reaching similar concentrations except for CoQ_9. Nonetheless, the relationship between CoQ_9 and the other antioxidants confirms that it had a similar behaviour pattern. This may indicate that in our study, plasma antioxidant status was normalized after the nutritional intervention with exogenous antioxidants and did not differ between the doses of flavonoids ingested. However, we did not find any relationship between CoQ_{10} and OJ intake that could explain the mechanism of its regulation. CoQ_{10} is well known to be part of the NEADS in both plasma and cells, originated from endogenous synthesis and food intake.

Additionally, we observed that 12-week intervention with either the NPJ or the HPJ induced a modification in the E-ADS. In particular, we found a decrease in catalase activity, which was also reported by Jain and Parmar (2011). This fact may be due to the scavenging activity of hesperidin, that may reduce superoxide anions and consequently the LPO and hydrogen peroxide generated during normal cell metabolism, thereby reducing the need for catalase biosynthesis (Wilmsen, Spada, and Salvador 2005). This is supported by the fact that the intake of hesperidin and narirutin were inversely correlated with catalase activity. Additionally, the presence of lower levels of LPO and hydrogen peroxide, which are substrate for the GPX, may generate less oxidised glutathione (Lobo *et al.* 2010); thus, the need for GR activity may have decreased, besides, this fact did not influence the glutathione-dependent antioxidant defence since the GSH and GSSG blood concentrations did not change after the interventions. In contrast, supplementation with OJ (i.e., hesperidin or narirutin) has been found to increase SOD activity (Niki 2014; Shi *et al.* 2012; Choi 2008) which may reduce the risk of MS (Li *et al.* 2013).

Several studies in animals reported a relationship between hesperidin, naringin and an increase in SOD gene expression (Choi 2008; Jeon *et al.* 2001). This finding is in accordance with our data in the HPJ intervention, and provides a possible explanation for the improved antioxidant status observed following the supplementation. In fact, Cilla *et al.* (2009) reported that the SOD induction observed after the consumption of a mixed fruit beverage (grape-orange-apricot) may be more effective than the accumulation of exogenous antioxidants in the plasma (Cilla *et al.* 2009). Notably, although GPX was not affected by OJ intake in the present study, we observed a relationship between SOD and GPX, which may represent an increase in erythrocyte cell membrane antioxidant defence. Additionally, a study developed in rats showed that the protective antioxidant role of hesperidin may be related to an improvement in membrane permeability, maintaining its structural integrity by protecting the ATPases from the deleterious effect of lipid peroxidation (Nandakumar *et al.* 2013).

Orange juice and inflammation

Evidence in literature had shown that flavanones possess anti-inflammatory properties, as reported on different experiments, *in vitro* and in animal models, that show a naringin-guided reduction of inflammatory biomarkers, such as CRP, TNF-α, IL-6, IL-8 and PAI-1 (Chanet *et al.* 2013; Choe *et al.* 2001; Jeon *et al.* 2001; Bodet *et al.* 2008; Devaraj *et al.* 2011). However, other authors reported no significant changes on inflammatory biomarkers when the use of flavanones was assessed (Habauzit *et al.* 2015; Morand *et al.* 2011; Devaraj *et al.* 2011). In this regard, we did not find a consistent evidence of the improvement of plasma inflammatory biomarkers (IL-1 and IL-6), since sometimes these effect are different depending on the intervention with NPJ and HPJ. Additionally, there were *P*-interactions on IL-1, IL-8 and TNF-α due to a significant better response to the intervention with the HPJ in the first arm of the study and a non-significant response during the second arm, which is probably associated to a carryover effect.

On the other hand, we had found an increase of galactonate, a compound derived from pectin present in orange peel. There is evidence that the consumption of citrus pectin could modulate inflammation and carcinogenesis (Bergman *et al.* 2010; Chen *et al.* 2006; Salman *et al.* 2008). However, we did not find clear association between the increase of galactonate and the decrease of inflammatory cytokines. Therefore, we cannot reach any conclusion related to the effect of OJ on inflammatory biomarkers.

With the metabolomic approach, we have determined possible changes in metabolites related to oxidative stress and inflammation. Polyunsaturated fatty acids are highly oxidised molecules and in addition, precursors of lipid mediators such as eicosanoids and prostaglandins that can regulate a variety of biological processes including inflammation, differentiation, angiogenesis and cellular proliferation (Calder 2006). We have found that the eicosanoid 5-HETE was elevated after the NPJ intervention, while 12-HETE was increased after the HPJ intervention compared to their respective baselines. 5-HETE is generated from arachidonate via the enzyme 5-lipoxigenase and is involved in the synthesis of leukotrienes and other proinflammatory mediators (Hao and Breyer 2007). By contrary, 12-HETE is generated from arachidonate by 12-lipoxigenase and can regulate vasoconstriction as well as counteract inflammation and tissue damage. In addition, during severe oxidative stress 12-HETE might offer a compensatory mechanism to maintain the functional integrity of platelets under these conditions (Porro *et al.* 2014). Moreover, Alpert *et al.* (2002) reported a protective role of 12-HETE by mediating the substrate regulation of the glucose-transport mechanism during hyperglycaemia. Specifically, the increased production rate of 12-HETE, mediates the down regulation of GLUT-1 expression and the glucose-transport system in vascular endothelial and smooth muscle cells (Alpert *et al.* 2002).

On the other hand, 9,10-DiHOME and 12,13 DiHOME are linoleic-derived products synthesized by neutrophils and macrophages with a toxic effect when accumulated in the cells (Thompson and Hammock 2007). The toxic effect attributed to these molecules is related to mitochondrial dysfunction, suppression of neutrophil respiratory burst activity, increased cell oxidative stress, vasodilation, and apoptosis (Thompson and Hammock 2007). The presence of 9,10 DiHOME and 12,13 DiHOME in serum was higher at baseline in the group receiving the HPJ, but they were decreased after the intervention, reaching similar levels that those found in the NPJ group. Interestingly, the inverse correlation found among these two molecules, 12, 13-DiHOME and 9,10-DiHOME, and ferulic acid 4-sulphate, which is a potent antioxidant that scavenges free radicals and enhances the cell stress response (Mancuso and Santangelo 2014), may influence the regulation of oxidative stress in subjects that received the HPJ.

Orange juice and the metabolic syndrome

We have also evaluated the influence of OJ intake on MS clinical signs. The decrease of BMI, WC and plasma leptin following the intake of either juice was due to the decrease in energy intake and was not associated with the flavanone supplementation. In fact, BMI, WC and plasma leptin were correlated with energy intake but not with urinary flavanones. To our best knowledge, there is no evidence that associates weight loss with flavanones (Galleano *et al.* 2012). On the contrary, the subjects in our study showed a significant decrease in DBP and SBP associated to the OJ flavanones intake, as previously reported (Morand *et al.* 2011). In fact, this hypotensive effect has been attributed to a NO-mediated vasodilatation mechanism (Yamamoto, Suzuki, and Hase 2008).

Besides that, the glucose and insulin concentrations presented at baseline and at the end of both interventions were between normal ranges, both OJs caused an increase in glucose and a decrease in insulin serum concentrations. The effect on glucose may been associated with either the daily intake of 500 ml of OJ, providing 56-70.5 g of CHO or may be a result of the lower insulin secretion found in our volunteers. On the other hand, the presence of stachydrine in the OJ may exert protective effect, since this compound has shown to counteract the detrimental effects of high-glucose by different mechanisms: 1) downregulating p16INK4^{A} protein levels and 2) by preventing the inhibition of SIRT1 activity and expression (Servillo *et al.* 2013). Additionally, the HOMA-IR index, which estimates the insulin resistance, was not modified after the intervention with either of the OJs supporting the neutral effect of both interventions on the carbohydrate metabolism. Other studies have attributed anti-diabetogenic properties to hesperidin and naringin administered alone to rats (Niki 2014). Therefore, more studies are needed to elucidate the effects of different doses of polyphenols on blood glucose, insulin secretion and whether the food matrix may modulate these effects.

Finally, in our study, supplementation with hesperidin and narirutin from OJs did not demonstrate significant changes on lipid metabolism, since only serum Apo-B levels were decreased after the 12-wk NPJ intervention. This neutral finding is in agreement with Demonty *et al.* (2010), who found that 4-wk intervention with either 500 mg/day of pure naringin or 800 mg/day of pure hesperidin did not have an effect on lipid metabolism in 204 moderately hyperlipidemic subjects. In addition, other studies have observed a decrease of Apo-B after OJ

or flavanones supplementation (Wilcox *et al.* 2001; Mulvihill *et al.* 2009; Sharma *et al.* 2012; Rizza *et al.* 2011). By contrast, other trials have shown that doses higher than 400 mg/day of hesperidin (administered either in OJ or as a pure compound) might improve the blood lipid profile (Kurowska *et al.* 2000; Miwa *et al.* 2005). It is noteworthy that in our study, baseline levels of blood TAG and LDL-C were lower compared to those observed by those previous studies. Therefore, more studies are needed in order to conclude about the effect of different flavanones on lipid metabolism.

In summary, our results show that the consumption of an OJ with at least 300 mg of flavanones over a 12-wk period enhanced the antioxidant defence system, protected against DNA damage and lipid peroxidation, and decreased BP in overweight and obese adults. The use of metabolomics could give a deeper insight in nutritional interventions, as we verified it is possible to determine new biomarkers to assess the validity of the dietary intervention and also go further and determine the effects on health and pathology that are not possible to find with traditional biomarkers, helping to provide a better dietary advice. It is necessary to expand the strategy to a greater population and validate the results obtained in the present Ph.D. thesis. In addition, the elucidation of the specific role of each flavanone and their mechanisms of action will require further studies.

CONCLUSIONS

CONCLUSIONS

I. Consumption of OJ with at least 300 mg of flavanones during 12-wk decreases oxidative biomarkers such as urinary 8-hydroxy-2'-deoxyguanosine and 8-iso-prostaglandin F2α.

II. Consumption of OJ with at least 300 mg of flavanones during 12-wk enhances the erythrocyte enzymatic antioxidant defence system by reducing erythrocyte catalase and glutathione reductase activities. Furthermore, high concentration of polyphenols increased the erythrocyte superoxide dismutase.

III. Consumption of OJ with at least 300 mg of flavanones during 12-wk did not modified the plasma non-enzymatic antioxidant defence system.

IV. Consumption of OJ with at least 300 mg of flavanones during 12-wk did not clearly improved inflammatory biomarkers.

V. Consumption of OJ with at least 300 mg of flavanones during 12-wk may help to improve BP in overweight and obese volunteers but it does not modify other components of the MS.

VI. A different metabolic signature is observed after 12-wk intervention with HPJ compared with the NPJ intervention. The presence of metabolites as betonicine and stachydrine, ferulic acid 4-sulphate and dihydroferulic acid appears to be new biomarkers related to OJ and polyphenols consumption.

VII. We have identified 9 and 13-HODE, 9,10-DiHOME and 12,13-DiHOME and 5-HETE and 12 HETE as metabolites that could help to determine the progression of oxidative and inflammatory status. Furthermore, the consumption of HPJ leads to a decrease in the concentration of 9 and 13-HODE, 9,10, 12,13-DiHOME, 5-HETE and an increase in 12 HETE, suggesting an enhancement of oxidative stress.

LIMITATIONS AND STRENGHTS OF THE STUDY

LIMITATIONS AND STRENGHTS OF THE STUDY

The following limitations should be taken into account. Firstly, the use of a control (placebo) group might strength our study design and give a further insight that help us to compare the results of the two different doses of polyphenols with free living subjects without the consumption of flavanones.

Secondly, a determinant confounding factor in crossover trials is the *carryover* effect that could be related to fatigue or short-term for washout and it is reflected on a different response in the two periods of intervention. In our study, we implemented a though statistical analysis (LMM) to manage adequately this effect but the significant P-interactions showed that there was still a carryover effect that avoids to see the real effects in some outcomes. Additionally, the inclusion of the LMM permits to avoid the presence of confounding factors such as BMI.

Thirdly, the small sample in the metabolomics analysis may limit the differences observed, i.e. the significant effects did not remain significant when adjusted by the FDR. The inclusion of a greater number of subjects might be crucial to increase the statistical power and determine the relationship in regards to the dietary intervention. Furthermore, the differences found when comparing the metabolic signature between genders open a new approach on how to organise the target groups. In our study, due to the limited sample, it was not viable to analyse the data nested by gender.

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ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
8-iso-PGF2α	Isoprostanes
ApoAI	Apolipoprotein AI
Apo-B	Apolipoprotein B
BMI	Body mass index
CoQ	Coenzyme Q
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FMD	Flow mediated dilation
GC-MS	Gas chromatography-mass spectrometry
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione reduced
GSSG	Glutathione oxidised
Hb	Haemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment
HPLC	High-performance liquid chromatography
HPJ	High polyphenols orange juice
IL-1	Interleukin-1
IL-6	Interleukin-6
IR	Insulin resistance
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-density lipoprotein
LPO	Lipid peroxides
MDA	Malondialdehyde
MS	Metabolic syndrome
NADPH	Nicotinamide adenine dinucleotide
NEADS	Non-enzymatic antioxidant system
NPJ	Normal polyphenols orange juice
OJ	Orange juice
oxLDL	Oxidised low density lipoprotein
PAI-1	Plasminogen activator inhibitor 1

PPARy	Peroxisome proliferator-activated receptor y
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SOD	Superoxide dismutase
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TNF-a	Tumour necrosis factor alpha
UPLC	Ultra-performance liquid chromatography
WC	Waist circumference
WHO	World health organization

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[QA1] Normal or High Polyphenol Concentration in Orange Juice Affects Antioxidant Activity, Blood Pressure, and Body Weight in Obese or Overweight Adults[1–3]

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Abstract

[AQ3] **Background:** The consumption of orange juice may lead to improved oxidative stress and may enhance the antioxidant defense system.

Objective: The aim was to evaluate the effects of the intake of orange juice containing either normal (NPJ) or high (HPJ) concentrations of polyphenols (299 and 745 mg/d, respectively) on the antioxidant defense system, oxidative stress biomarkers, and clinical signs of metabolic syndrome in 100 nonsmoking subjects who were either overweight or obese.

Methods: A randomized, double-blind crossover study was conducted over two 12-wk periods with a 7-wk washout period. The effects on enzymatic and nonenzymatic blood antioxidant defense system, urinary and plasma oxidative stress biomarkers, and clinical signs of metabolic syndrome were evaluated before and after an intervention with both of the orange juices. Paired *t* tests and linear mixed-effects models were used to evaluate the effects of juice, time, and interactions.

Results: The intake of either NPJ or HPJ led to a decrease in urinary 8-hydroxy-2′-deoxyguanosine (NPJ: 935 ± 134 to 298 ± 19 ng/ [AQ4] mg creatinine; HPJ: 749 ± 84 to 285 ± 17 ng/mg creatinine), 8-iso-prostaglandin F2a (NPJ: 437 ± 68 to 156 ± 14 ng/mg creatinine; HPJ: 347 ± 43 to 154 ± 13 ng/mg creatinine), erythrocyte catalase, and glutathione reductase activities. A decrease was also observed in body mass index, waist circumference, and leptin (all $P < 0.05$). The NPJ group showed decreased systolic and diastolic blood pressures (systolic blood pressure: 128 ± 1 to 124 ± 2 mm Hg; diastolic blood pressure: 79 ± 1 to 76 ± 1 mm Hg), whereas the HPJ group showed increased erythrocyte superoxide dismutase (SOD) activity (17.7 ± 1.5 to 23.1 ± 1.7 U/mg hemoglobin).

Conclusions: Our results show that the consumption of either NPJ or HPJ protected against DNA damage and lipid peroxidation, modified several antioxidant enzymes, and improved body weight in overweight or obese nonsmoking adults. Only blood pressure and SOD activity were influenced differently by the different flavanone supplementations. This trial was registered at clinicaltrials.gov as NCT01290250. *J Nutr* 2015;145:1–9.

[AQ5] **Keywords:** antioxidants, flavanones, obesity, oxidative stress

Introduction

Reactive oxygen species consist of a variety of structures, including both free radicals and nonradicals, that are generated by normal cellular metabolism and by exogenous agents that may serve as cell signaling molecules or bactericidal agents. An excess of reactive oxygen species formation causes extensive damage to cellular macromolecules such as polyunsaturated

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[3] Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at https://jn.nutrition.org.
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lipids, proteins, and DNA and leads to a process known as oxidative stress, which is associated with cardiovascular disease and related risk factors, such as obesity, type 2 diabetes, cancer, and other aging-related diseases (1).

Flavonoids are phenolic compounds that may be found in fruit, vegetables, and plant-derived beverages. They are powerful in vitro antioxidants with pharmacologic properties similar to those of antithrombotic, antifibrotic, and anti-inflammatory drugs (2, 3). Their consumption has been shown to be inversely associated with morbidity and mortality from coronary heart diseases (4). In particular, orange juice (OJ)[12] contains flavanone glycosides (e.g., hesperidin and narirutin), which are a subgroup of flavonoids mainly present in the solid parts of fruit (e.g., the albedo) and the membranes separating the pulp segments, which explains the higher concentrations of flavanones in whole-fruit juices. Flavanones are deglycosylated in the colon by intestinal microflora; this produces the active aglycones hesperitin and naringenin, which possess similar chemical structures (5). Both glycosides may have anti-inflammatory, hypolipidemic, and vasoprotective properties that may lead to beneficial effects on blood pressure (BP) and LDL-cholesterol control (6, 7). However, although polyphenols are known to be antioxidants, the pro-oxidant effects of high doses have also been reported (8). Some flavonoids may auto-oxidize and produce superoxide and hydrogen peroxide free radicals, and it has been shown that this flavonoid-induced DNA damage may be due to the generation of hydroxyl or other free radicals (9–11). Therefore, it is difficult to reach a consensus on the adequate doses of these compounds for the prevention of diseases. The present study aimed to evaluate the effects of the intake of OJ containing either normal or high concentrations of flavanones on the antioxidant defense system, oxidative stress biomarkers, and clinical signs of metabolic syndrome in overweight and obese adult volunteers.

Methods

Study design. A randomized, crossover, double-blind (subjects and investigators), 12-wk dietary intervention trial was conducted with the use of OJ containing the following 2 polyphenol amounts: *1*) 0.6 g/L [OJ with a normal polyphenol concentration (NPJ)] and *2*) 1.5 g/L [OJ with a high polyphenol concentration (HPJ)]. The advantage of using a crossover design is that each subject served as his or her own control. A 7-wk washout period was used between the 12-wk consumption of each juice. The subjects were randomly assigned to each of the 2 groups, which were paired according to sex and age, by using a random-number generator program. The first group (n = 54) received 2 daily doses (250 mL each) of the HPJ for 12 wk (corresponding to a daily dose of 582.5 mg hesperidin, 125 mg narirutin, and 34 mg didymin; **Supplemental Table 1**). After a 7-wk washout period, the subjects received the NPJ daily (corresponding to 237 mg hesperidin, 45 mg narirutin, and 17 mg didymin; Supplemental Table 1). The second group (n = 46) received the NPJ for 12 wk followed by a 7-wk washout period, after which they received the HPJ for 12 wk. After the efficacy of the washout period was verified by confirming that the initial baseline and postwashout baseline data were similar, we merged the results from both arms of the study (the HPJ and NPJ interventions; n = 100 each) to analyze the data. Thus, our data are presented according to 2 periods. The HPJ period includes the data derived from all of the subjects who consumed the HPJ during the 2 arms of the intervention, and the NPJ period includes the results obtained from all of the subjects who consumed the NPJ.

Subject selection and allocation. **Figure 1** shows a flow diagram of the selection, allocation, and crossover randomization of the subjects involved in the study. Participants were recruited through local newspaper advertisements. Among the ~500 volunteers who were screened, 210 (aged 18–65 y) were deemed eligible and were enrolled in the study. The sample number was estimated by considering the specific variances of the methodology for all of the variables with a type I error $\alpha = 0.05$ and a type II error $\beta = 0.2$ (80% power). The inclusion criteria were a BMI (in kg/m^2) ≥25 (overweight or obese) but <40 (extreme obesity) or a waist circumference (WC) >94 cm for men and >80 cm for women. The exclusion criteria were as follows: the presence of morbid obesity (BMI ≥40), diastolic BP (DBP) ≥110 mm Hg; fasting plasma glucose concentration ≥130 mg/dL; the use of any medication for the control of BP, glucose, or lipid metabolism; a medical history of hypocaloric diet consumption within the past year; gastrointestinal tract disorders; and the presence of familial dyslipidemias in blood relatives or refusal to take part in the study. As a complication of being overweight or obese, most of the subjects had alterations in at least 1 clinical sign of metabolic syndrome, including hypertension (DBP ≥85 and <110 mm Hg), hyperglycemia (≥100 and <130 mg/dL), elevated plasma TG concentrations (≥150 mg/dL), and decreased plasma HDL-cholesterol concentrations (<40 mg/dL for men and <50 mg/dL for women), as established by the International Diabetes Federation (12).

Fifty-nine subjects were unable to complete the study mainly due to the long intervention period and to self-reported gastric acidity that was related to their OJ intake; thus, a total of 151 volunteers completed the intervention. We targeted a general population; however, due to the high impact of smoking on oxidative stress status and the antioxidant defense system (13), we selected only nonsmoking subjects for the analysis (n = 100).

Study performance. During the time that the volunteers participated in the study, they made 4 visits to the clinical research unit: 1 at baseline,

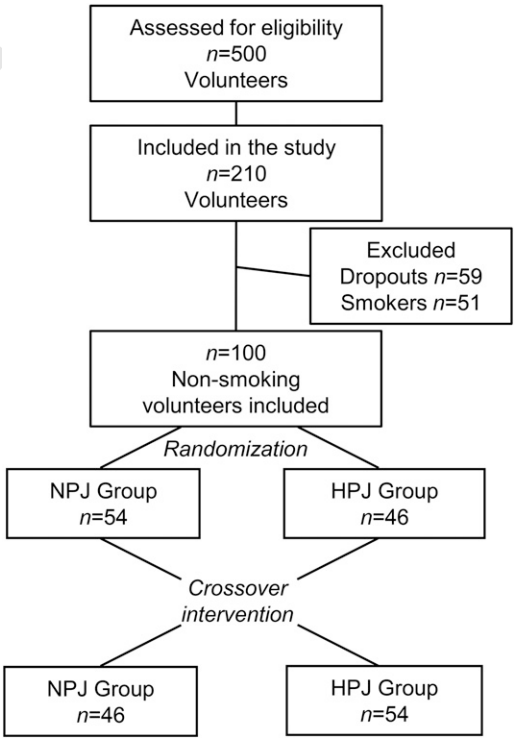

FIGURE 1 CONSORT (Consolidated Standards of Reporting Trials)-based flow diagram of the recruitment, enrollment, and randomization process. HPJ, high-polyphenol-concentration orange juice; NPJ, normal-polyphenol-concentration orange juice.

[12] Abbreviations used: BP, blood pressure; CoQ, coenzyme Q; DBP, diastolic blood pressure; GPX, glutathione peroxidase; GR, glutathione reductase; GSSG, oxidized glutathione; HPJ, high-polyphenol-concentration orange juice; LPO, lipid peroxide; NEADS, nonenzymatic antioxidant system; NPJ, normal-polyphenol-concentration orange juice; OJ, orange juice; oxLDL, oxidized LDL; SOD, superoxide dismutase; SBP, systolic blood pressure; WC, waist circumference; 8-iso-PGF_{2a}, 8-isoprostane prostaglandin F2a; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

1 after the washout period, and 1 each at the end of both intervention periods. Anthropometric measurements, biological sample collection, and a dietary interview, which included an FFQ (14), were conducted during the visits. The participants were asked to maintain their usual diet and to avoid heavy physical activity, alcohol use (24 h), and smoking (2 h) before the visits. Once the intervention period commenced, all of the volunteers were asked to consume the same calories as during their usual diet to prevent weight gain or loss during the study period. To aid them in balancing out the additional calories that they were ingesting from OJ intake, the volunteers received nutritional advice.

The dietary intake of the participants was assessed by using an FFQ at the beginning and at the end of each period. The data were processed [AQ7] with the use of CSG software (General ASDE) and the Spanish Food Composition Database (15).

All study procedures were approved by the University Hospital Virgen de las Nieves Ethics Committee. The study was conducted according to the principles of the Declaration of Helsinki, and all of the volunteers gave written informed consent before the start of the intervention. This trial was registered at clinicaltrials.gov as NCT01290250.

Products. Both the NPJ and HPJ were made from fresh fruit and provided by Coca Cola Europe. For the reference NPJ, a commercially available product with a normal amount of polyphenols was used (299 mg in 500 mL/d; Minute Maid). The HPJ (Minute Maid, Whole Press) was enriched with polyphenols that were extracted from orange albedo and pulp by a patented method and was also commercially available (745 mg in 500 mL/d). Thus, the HPJ contained twice the phytocompounds of NPJ. The composition of both juices is detailed in Supplemental Table 1.

Anthropometric characterization. A single trained examiner obtained the anthropometric measurements while participants were barefoot and wearing only nonrestrictive undergarments. A bioelectrical impedance analyzer (Tanita Europe BV) was used to determine body weight (in kg) [AQ8] and body composition. A scale (Anó Sanyol) was used to measure height (in cm), and a flexible tape (Holtain Ltd.) was used to measure WC (in cm). All of the measurements were obtained by using standardized procedures and periodically calibrated instruments.

BP was measured according to standard methods by using a validated oscillometric technique (Omron M4-I Intellisense; Omron Corporation). BP was determined in the nondominant upper arm after a 20-min resting period. Three values were taken at 2-min intervals, and the average of these measures was considered.

Blood and urine sampling. Blood samples were collected in the fasting state between 0800 and 1100 h, and the samples were immediately [AQ9] centrifuged. Serum and plasma specimens and blood erythrocytes were frozen at −80°C until further analysis. First-morning urine was collected in 3 containers and stored at −80°C until subsequent analyses.

Determination of general serum biochemical variables. Serum concentratons of glucose, TGs, HDL cholesterol, LDL cholesterol, apo A-I, and apoB were measured by using the clinical analysis system Roche Hitachi Modular DPP (Roche Diagnostics Spain, S.L.). Fasting insulin was determined in samples by using an Elecsys Modular E-170 (Roche Diagnostics Spain, S.L). The HOMA-IR was calculated with the equation HOMA-IR = fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5.

Determination of polyphenols in urine. The determination of both hesperetin and naringenin and their metabolites was performed by using [AQ10] a UHPLC system (1290 InfinitySeries; Agilent Technologies), which was equipped with a triple quadrupole mass spectrometer (6460 series; Agilent Technologies). A Poroshell 120 C18 column (100 × 3.0 mm i.d., 2.7 μm; Agilent Technologies) was used at 30°C, and the injected volume was 2 μL. Gradient elution was performed by using water/formic acid (99:0.1, vol:vol) and acetonitrile/formic acid (99:0.1, vol:vol) at a constant flow rate of 0.5 mL/min. The gradient commenced with the following proportions (vol:vol) of acetonitrile [*t* (min), % acetonitrile]: (0, 1), (10, 40), (12, 100), and (14, 1), respectively, followed by 1 min of [AQ11] post-time, as previously reported by Tomás-Navarro et al. (16). The concentrations of hesperetin diglucuronide, hesperetin sulfoglucuronide, naringenin diglucuronide, and naringenin sulfoglucuronide metabolites were estimated by using calibration curves with synthetic flavanones as standards (20, 10, 5, 2.5, 1 μM). [AQ12]

The intraday repeatability of the UHPLC-QqQ method was assessed [AQ13] from 10 consecutive chromatographic runs by using a standard solution with 2.5 μM of each standard in methanol containing 0.1% (vol:vol) formic acid. The interday repeatability of the method was assessed by analyzing the same standard solution for 2 consecutive days. The relative SDs for the peak area ranged from 0.5% to 4.7% on the intraday test and 1.3% to 3.5% on the interday test.

Determination of erythrocyte enzymatic antioxidant activities. The hemoglobin concentration in the blood samples was spectrophotometrically determined by the colorimetric cyanmethemoglobin method (17) by using Sigma Diagnostic reagents. Erythrocyte antioxidant enzyme activities, including catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR), were spectrophotometrically assayed in a plate reader (Synergy HT; Biotek Instruments) and expressed as nmol/(L • g hemoglobin), U/mg hemoglobin, U/g hemoglobin, and U/g hemoglobin, respectively (18–21). The protocols were adapted to micromethods.

Determination of plasma nonenzymatic antioxidant concentrations. After extraction with 1-propanol, plasma concentrations of [AQ14] α-tocopherol, retinol, coenzyme Q (CoQ) 9, and CoQ_{10} were determined by HPLC coupled with an electrochemical detector. β-Carotene was determined after extraction with 1-propanol by using an HPLC system attached to a multi-wavelength UV detector set at 450 nm. All of the compounds were identified by comparing their retention times with the predetermined retention times of the individual standards. Concentrations are given as micrograms per milliliter of plasma.

Determination of urinary 8-isoprostane prostaglandin F2a and 8-hydroxy-2′-deoxyguanosine concentrations. Urinary samples were analyzed for 8-isoprostane prostaglandin F2a (8-iso-PGF_{2a}) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) by using commercial competitive ELISA kits (Oxford Biomedical Research and JAICA, respectively) with determination ranges of 0–75 μg/L and 0.125–10 μg/L, respectively (CVs: 7.2% and 8.3%, respectively). The concentrations were normalized by using urinary creatinine: the 8-iso-PGF_{2a}:creatinine and 8-OHdG:creatinine ratios were calculated and expressed in nanograms per milligram of creatinine. Creatinine concentration was determined at the CEBAS by using an UHPLC system (1290 Infinity Series; Agilent [AQ15] Technologies) coupled with an electrochemical detector.

Determination of plasma oxidized LDL, lipid peroxide, and malondialdehyde. A commercial ELISA kit (Biomedica) was used to determine oxidized LDL (oxLDL; determination range: 0–750 μg/L; CV: 11.8%). A colorimetric commercial assay kit (Oxystat; Biomedica) was used to determine the total lipid peroxides (LPOs; detection limit: 7 μmol/L; CV: 8.3%). Plasma malondialdehyde was determined by using a TBARS assay kit (OxiSelect; Cell Biolabs; determination range: 0–125 μM; CV: 2.15%). Absorbance was measured in a plate reader (Synergy HT; Biotek Instruments).

Statistical analysis. Values are presented as means ± SEMs. Before statistical analyses, the data were assessed for normality by using the Shapiro-Wilk test. The homogeneity of the variances was estimated by using Levene's test. An independent-samples *t* test was conducted to identify possible differences between the groups who started with either NPJ (n = 46) or HPJ (n = 54) at baseline. To verify the efficacy of the washout period, a paired *t* test was conducted between the initial baseline data and the postwashout baseline data. Once the efficacy of the washout period was verified, the initial baseline and postwashout baseline data were combined and the final data from each study arm (n = 100 each for the 2 interventions) were combined. Then, paired-sample *t*

tests were conducted between the basal and postintervention data to determine the effects of each intervention on each variable.

A linear mixed-effects model, with the intercept as a random effect and a covariance structure for repeated measures by time and OJ, was used to determine the differences between the interventions (i.e., intervention effects). This model allows for the heterogeneity of the outcome responses between juice intake at each time point and at the individual level of the participants. Note that the baselines were also treated as outcome responses but without the associated juice intake effects. Therefore, in the model, time was included as a categorical variable indicating the beginning and end of each arm of the trial. Furthermore, the fixed effects included time, juice intake, age, sex, BMI, WC, energy intake, and the interaction between juice intake and the arms of the intervention (i.e., *P*-interaction) and were used as covariates to adjust for possible confounding factors. Energy intake did not have an impact on any variable and was eliminated from the models.

Correlations between the concentrations of the main flavanones and variables were estimated by the Pearson's correlation coefficient when the assumptions of normality were met and by the Spearman's correlation coefficient when the assumptions of normality were not met. $P < 0.05$ was considered significant. All of the statistical analyses were performed by using SPSS 20.0 (IBM Corporation) for Windows.

Results

With the exception of the decrease in energy intake that occurred during the intervention with both of the OJs, there were no significant differences in dietary intake (i.e., energy, macronutrients, and fiber) between the 2 interventions after the 12-wk period (**Supplemental Table 2**).

Preintervention characteristics of subjects by group. Before the intervention, the anthropometric, body composition, and biochemical variables were similar between the 2 groups of volunteers, with the exception of DBP ($P = 0.049$), as shown in **Table 1**.

Baseline characteristics before the intervention with each OJ are included in **Tables 2** and **3**. Weight, WC, BP, body composition, serum glucose and lipid metabolism biomarkers, and plasma leptin (Table 2) and urine polyphenols, erythrocyte antioxidant enzymes, plasma retinol, and oxidative biomarkers (Table 3) did not differ between the 2 baseline periods. In contrast, plasma concentrations of α-tocopherol, CoQ_9, and CoQ_{10} were lower before the HPJ intervention ($P \leq 0.026$), whereas the β-carotene concentration was higher before the HPJ intervention ($P \leq 0.001$) than before the NPJ period (Table 3).

Effects of OJ intake on measures of anthropometry, glucose and lipid metabolism, and leptin. As shown in Table 2, weight, BMI, and WC decreased after the intake of both juices (all $P \leq 0.001$), and systolic BP (SBP) and DBP decreased only after NPJ intake ($P = 0.009$ and $P \leq 0.001$, respectively). In addition, BMI and WC were correlated with energy intake, SBP, leptin, 8-OHdG, and 8-iso-PGF_{2a} (**Table 4**). Nevertheless, there were no significant differences in the anthropometric variables in the participants between the 2 interventions.

Glucose increased significantly after the intake of either of the juices ($P \leq 0.001$), and plasma insulin concentrations decreased after NPJ intake ($P = 0.04$) and tended to decrease after HPJ intake ($P = 0.06$). However, the juice effects on glucose, insulin, and the HOMA-IR index were significantly higher (glucose: β = 0.017 mmol/L; insulin: β = 1.31 μU/mL; HOMA-IR: β = 0.38) after HPJ intake ($P = 0.009$, 0.007, and 0.040, respectively) than after NPJ intake. With regard to lipid metabolism, total, HDL-, and LDL-cholesterol concentrations were similar throughout the study, with no significant difference between NPJ and HPJ intake. Plasma TGs and apoB concentrations decreased significantly only after the intake of the NPJ ($P = 0.049$ and $P \leq 0.001$, respectively), whereas apo A-I significantly increased after HPJ intake ($P = 0.024$). Moreover, plasma leptin decreased after the 12-wk intervention with either the NPJ or the HPJ ($P = 0.007$ and 0.02, respectively; Table 2).

TABLE 1 Anthropometric characteristics and body composition, serum biochemistry, plasma leptin, urine polyphenols, and dietary intakes of overweight and obese adults before the HPJ and NPJ interventions[1]

	NPJ	HPJ
Weight, kg	93.3 ± 2.5	91.4 ± 1.9
BMI, kg/m^2	33.1 ± 0.6	33.2 ± 0.5
WC, cm	105 ± 2	102 ± 1
SBP, mm Hg	132 ± 2	129 ± 2
DBP, mm Hg	83 ± 1	79 ± 1*
Body composition		
Body water, kg	43.1 ± 1.3	41 ± 1.1
Water, %	46.2 ± 0.7	44.7 ± 0.7
Body fat, kg	34.1 ± 1.4	34.7 ± 1.2
Body fat, %	37.5 ± 1.3	37.8 ± 1
Lean mass, kg	59.1 ± 1.6	56.9 ± 1.5
Muscle mass, kg	55.8 ± 1.6	54.1 ± 1.4
Bone mass, kg	3.0 ± 0.1	2.9 ± 0.1
Glucose and lipid metabolism		
Glucose, mmol/L	4.8 ± 0.1	4.7 ± 0.1
Insulin, μU/mL	14.9 ± 1.2	15.7 ± 1.4
HOMA-IR	3.3 ± 0.3	3.5 ± 0.4
TC, mg/dL	211 ± 5	217 ± 5
HDL cholesterol, mg/dL	49 ± 2	50 ± 2
LDL cholesterol, mg/dL	127 ± 4	133 ± 4
TGs, mg/dL	142 ± 9	137 ± 9
apo A-I, mg/dL	147 ± 3	147 ± 3
apoB, mg/dL	96 ± 3	100 ± 3
Uric acid	5.6 ± 0.2	5.2 ± 0.2
Leptin, pg/mL	23.2 ± 2.2	24.3 ± 2.2
Urine hesperidin, mg/L	0.83 ± 0.36	1.10 ± 0.41
Urine naringenin, mg/L	0.46 ± 0.20	0.38 ± 0.23

[1] Values are means ± SEMs; $n = 46$ for NPJ and $n = 54$ for HPJ. *Different from NPJ, $P < 0.05$. DBP, diastolic blood pressure; HPJ, high-polyphenol-concentration orange juice; NPJ, normal-polyphenol-concentration orange juice; SBP, systolic blood pressure; TC, total cholesterol; WC, waist circumference.

Polyphenols in urine. The presence of urinary flavanones was very low at baseline, as shown in Table 3. Urine hesperetin and naringenin metabolites increased after the intake of both juices (all $P \leq 0.001$), but they were significantly higher after the HPJ intervention than after the NPJ intervention ($P \leq 0.001$). Moreover, when analyzing the urinary concentration of both flavanones, we observed a significant interaction between the interventions and the arms of the study (i.e., *P*- interaction: all $P \leq 0.001$); the increase in flavanones was more pronounced during the first arm than during the second arm of the intervention (**Figure 2**A, B).

Erythrocyte antioxidant enzyme activities. Intervention with either HPJ or NPJ induced a similar decrease in GR ($P \leq 0.001$ and $P = 0.031$, respectively) and catalase activities ($P \leq 0.001$ for each). In contrast, SOD activity was significantly higher ($P = 0.008$) after HPJ consumption (Table 3). However, the erythro-

TABLE 2 Anthropometric characteristics, serum glucose, and lipid metabolism variables and plasma leptin concentrations in overweight and obese adults at baseline and after the 12-wk NPJ and HPJ interventions[1]

	NPJ		HPJ		
	Baseline	Final	Baseline	Final	*P* (intervention effect)
Weight, kg	90.4 ± 1.5	89.1 ± 1.5*	90.6 ± 1.5	88.8 ± 1.5*	NS
BMI, kg/m^2	32.5 ± 0.4	32.0 ± 0.4*	32.6 ± 0.4	31.9 ± 0.4*	NS
WC, cm	99.1 ± 1.3	95.1 ± 1.2*	99.4 ± 1.1	95.6 ± 1.1*	NS
SBP, mm Hg	128 ± 1	124 ± 2*	127 ± 1	124 ± 1	NS
DBP, mm Hg	79 ± 1	76 ± 1*	78 ± 1	77 ± 1	NS
Glucose, mmol/L	4.9 ± 0.1	5.2 ± 0.1*	5.0 ± 0.1	5.2 ± 0.1*	0.009
Insulin, μU/mL	12.7 ± 0.7	11.5 ± 0.6*	13.8 ± 0.9	12.7 ± 0.7	0.007
HOMA-IR	2.9 ± 0.2	2.7 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	0.004
TC, mmol/L	5.6 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	NS
HDL cholesterol, mmol/L	1.29 ± 0.03	1.32 ± 0.03	1.32 ± 0.03	1.29 ± 0.03	NS
LDL cholesterol, mmol/L	3.39 ± 0.08	3.47 ± 0.08	3.41 ± 0.08	3.49 ± 0.08	NS
TGs, mmol/L	1.49 ± 0.07	1.40 ± 0.07*	1.54 ± 0.07	1.47 ± 0.06	NS
apo A-I, mg/dL	147 ± 2	147 ± 2	149 ± 2	145 ± 2*	NS
apoB, mg/dL	95 ± 2	91 ± 2*	96 ± 2	93 ± 2	NS
Leptin, pg/mL	22.7 ± 1.5	19.6 ± 1.4*	22.9 ± 1.9	20.6 ± 1.6*	NS

[AQ29] [1] Values are means ± SEMs; $n = 100$ each for NPJ and HPJ. *Different from baseline, $P \leq 0.05$; #different from NPJ, $P < 0.05$ (intervention effect). No significant *P*-interactions were observed. DBP, diastolic blood pressure; HPJ, high-polyphenol-concentration orange juice; NPJ, normal-polyphenol-concentration orange juice; SBP, systolic blood pressure; TC, total cholesterol; WC, waist circumference.

[AQ30] [2] Interaction between juice intake effects and period was not significant in any of the variables presented in this table.

cyte antioxidant enzyme activities and the related antioxidant molecules reduced glutathione (GSH) and oxidized glutathione [AQ16] (GSSG) did not show any differences between the interventions. In addition, no significant differences were observed in these enzymes by either the interventions or the *P*-interactions.

Erythrocyte catalase activity was inversely correlated with hesperetin, naringenin, and GR, whereas it was positively correlated with 8-OHdG and 8-iso-PGF_{2a} (Table 4). In contrast, SOD activity was positively correlated with GPX and GR (Table 4).

Nonenzymatic antioxidant defense system. At baseline, CoQ_9 and CoQ_{10} plasma concentrations were lower and the β-carotene concentration was higher after the HPJ intervention than after the NPJ intervention (all $P \leq 0.05$; Table 3). Initial and final data showed an increase in CoQ_{10} ($P = 0.019$) after HPJ intake, whereas an increase in β-carotene was observed after NPJ intake ($P = 0.001$). After adjusting for age and sex, we observed higher plasma concentrations of retinol ($P = 0.009$), α-tocopherol ($P = 0.004$), β-carotene ($P = 0.008$), and CoQ_{10} ($P = 0.001$) in older subjects; male subjects had higher concentrations of retinol ($P \leq 0.001$) and CoQ_{10} ($P \leq 0.001$). There was a significant difference between groups for plasma CoQ_9 ($P = 0.002$) but not for plasma retinol, α-tocopherol, β-carotene, and CoQ_{10}. In addition, increased BMI was associated with lower plasma β-carotene concentrations (Table 4). The concentrations of these variables, with the exception of CoQ_9 ($P = 0.008$), were similar at the end of the NPJ and HPJ interventions. Finally, CoQ_9 was correlated with CoQ_{10} (Table 4).

Oxidative stress biomarkers. As presented in Table 3, urinary 8-OHdG and 8-iso-PGF_{2a} decreased after the 12-wk NPJ and HPJ interventions (all $P \leq 0.001$). In addition, urinary 8-OHdG was lower after HPJ intake than after NPJ intake ($P = 0.012$). Indeed, there was a significant *P*-interaction between urinary 8-OHdG and the study arms ($P = 0.002$); during the first arm of the intervention, NPJ intake caused a greater decrease in 8-OHdG than did HPJ intake ($P = 0.01$), whereas in the second study arm, NPJ intake induced an increase and HPJ induced a decrease in 8-OHdG ($P = 0.012$) (Figure 2C). There was no significant difference in 8-iso-PGF_{2a} concentrations between the 2 interventions. In addition, we observed a positive correlation between 8-OHdG and 8-iso-PGF_{2a} and negative correlations between 8-OHdG and CoQ_9 and between 8-OHdG and CoQ_{10} (Table 4). After adjusting for age, we observed that urine 8-iso-PGF_{2a} concentrations were higher in younger subjects and diminished with increasing age. On the contrary, plasma LPO decreased after the NPJ intervention ($P = 0.002$) but was maintained after the HPJ intervention. A significant difference in LPO was observed between the 2 interventions ($P = 0.003$), and the LPO values were lower in men than in women ($P \leq 0.001$). Moreover, LPO was inversely correlated with α-tocopherol (Table 4).

Finally, there were no juice intake effects on plasma malondialdehyde and oxLDL concentrations, and the concentrations did not significantly differ between the 2 interventions; however, we found that plasma malondialdehyde was lower in women ($P \leq 0.001$). In additionally, we observed a relation between hesperetin and malondialdehyde (Table 4).

Discussion

The present study demonstrates that a 12-wk intervention with OJ, regardless of its polyphenol concentration (299 or 745 mg/d), improves the antioxidant defense system and thus may reduce initial lipid peroxidation products (i.e., plasma LPO) and advanced peroxidation products (i.e., urinary 8-iso-PGF_{2a} and 8-OHdG). These findings indicate lower lipid- and DNA-oxidative damage after intervention in nonsmoking subjects who were either overweight or obese. Urinalysis demonstrated the presence of flavanone derivatives, which confirmed the subjects' adherence to the intervention. To the best of our

TABLE 3 Urinary polyphenols, erythrocyte antioxidant enzymes, plasma NEADS variables, and urinary oxidative stress biomarkers in overweight and obese adults at baseline and after the 12-wk NPJ and HPJ interventions[1]

	NPJ		HPJ		*P*	
	Baseline	Final	Baseline	Final	Intervention effect[2]	Interaction[3]
Flavanone conjugates in urine, mg/L						
Hesperitin	0.5 ± 0.2	19.5 ± 3.7*	0.8 ± 0.2	31.3 ± 6.5*	0.001	0.001
Naringenin	0.4 ± 0.15	3.8 ± 0.6*	0.5 ± 0.3	5.6 ± 1.2*	0.001	0.001
Erythrocyte antioxidant enzymes						
Catalase, nmol/(L · g Hb)	0.26 ± 0.01	0.23 ± 0.01*	0.26 ± 0.01	0.22 ± 0.01*	NS	NS
SOD, U/mg Hb	20.7 ± 1.6	21.1 ± 1.7	17.7 ± 1.5	23.1 ± 1.7*	NS	NS
GPX, U/g Hb	16.0 ± 0.4	15.5 ± 0.6	15.5 ± 0.4	15.3 ± 0.6	NS	NS
GR, U/g Hb	2.5 ± 0.1	2.11 ± 0.1*	2.33 ± 0.1	2.18 ± 0.1*	NS	NS
Plasma NEADS variables						
Retinol, μg/mL	0.24 ± 0.01	0.27 ± 0.01*	0.24 ± 0.01	0.26 ± 0.01	NS	NS
α-Tocopherol, mmol/L	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01*	NS	NS
α-Tocopherol/TC, μmol/mmol	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	NS	NS
α-Tocopherol/TG, μmol/mmol	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01*	NS	NS
β-Carotene, μg/mL	0.39 ± 0.03	0.68 ± 0.05*	0.65 ± 0.04#	0.63 ± 0.04	NS	NS
CoQ_9, μg/mL	7.3 ± 0.2	4.7 ± 0.4	5.5 ± 0.3#	6.1 ± 0.2	0.002	NS
CoQ_{10}, μg/mL	340 ± 9	300 ± 11	270 ± 10#	310 ± 10*	NS	NS
GSH, ppm	5.8 ± 0.7	6.3 ± 0.8	7.6 ± 0.8	6.1 ± 0.8	NS	NS
GSSG, ppm	42 ± 3	44 ± 3	40 ± 3	46 ± 3	NS	NS
Oxidative stress biomarkers						
Urine 8-iso-PGF_{2a}, ng/mg creatinine	437 ± 68	156 ± 14*	347 ± 43	154 ± 13*	NS	NS
Urine 8-OHdG, ng/mg creatinine	935 ± 134	298 ± 19*	749 ± 84	285 ± 17*	0.012	0.002
Plasma oxLDL, pg/mL	335 ± 40	343 ± 38	322 ± 39	326 ± 40	NS	NS
Plasma LPOs, μmol/L	0.75 ± 0.03	0.69 ± 0.03*	0.75 ± 0.03	0.77 ± 0.03	0.003	NS
Plasma malondialdehyde, μmol/L	22 ± 1	21 ± 1	20 ± 1	20 ± 1	NS	NS

[1] Values are means ± SEMs; $n = 100$ for NPJ and HPJ. #Different from NPJ at baseline, $P < 0.05$ (independent-samples *t* test); *different from baseline, $P < 0.05$. NS, $P > 0.05$. CoQ, coenzyme Q; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; Hb, hemoglobin; HPJ, high-polyphenol-concentration orange juice; NPJ, normal-polyphenol-concentration orange juice; LPO, lipid peroxide; NEADS, nonenzymatic antioxidant defense system; oxLDL, oxidized LDL; ppm, parts per million; SOD, superoxide dismutase; TC, total cholesterol; 8-iso-PGF_{2a}, 8-isoprostane prostaglandin F2a; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.
[2] Indicates differences between the 2 interventions with the linear mixed model, $P < 0.05$.
[3] Indicates interaction between the interventions and the arms of the study, $P \leq 0.05$.

knowledge, this is the first long-term, large-scale crossover intervention trial to evaluate the effects of citrus juices with different doses of flavanones (mainly hesperidin and narirutin conjugates) on the antioxidant defense system and oxidative stress biomarkers in overweight and obese adults. There is limited evidence confirming that polyphenol-rich products

TABLE 4 Correlation between biochemical variables in all overweight and obese adults after the 12-wk NPJ or HPJ interventions[1]

	BMI		WC		Catalase		SOD		LPOs		Malondialdehyde		8-OHdG		CoQ9	
	r	*P*	*r*	*P*	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*
Energy intake	0.39	≤0.001	0.26	≤0.001	—	—	—	—	—	—	—	—	—	—	—	—
SBP	0.29	≤0.001	0.25	≤0.001	—	—	—	—	—	—	—	—	—	—	—	—
DBP	0.28	≤0.001	0.23	≤0.001	—	—	—	—	—	—	—	—	—	—	—	—
Leptin	0.27	≤0.001	0.31	≤0.001	—	—	—	—	—	—	—	—	—	—	—	—
Hesperidin	—	—	—	—	−0.17	0.013	—	—	—	—	0.16	0.013	—	—	—	—
Naringenin	—	—	—	—	−0.19	0.005	—	—	—	—	—	—	—	—	—	—
GR	—	—	—	—	−0.15	0.015	0.15	0.02	—	—	—	—	—	—	—	—
GPX	—	—	—	—	—	—	0.29	≤0.001	—	—	—	—	—	—	—	—
CoQ9	—	—	—	—	—	—	—	—	—	—	—	—	−0.17	0.016	—	—
CoQ10	—	—	—	—	—	—	—	—	—	—	—	—	−0.17	0.023	0.63	≤0.001
α-Tocopherol	—	—	—	—	—	—	—	—	−0.17	0.016	—	—	—	—	—	—
8-OHdG	0.26	≤0.001	0.23	≤0.001	0.29	≤0.001	—	—	—	—	—	—	—	–	—	—
8-iso-$PGF_{2\alpha}$	0.23	≤0.001	0.22	≤0.001	0.25	≤0.001	—	—	—	—	—	—	0.72	≤0.001	—	—

[1] Values are Pearson's (*r*) or Spearman's (ρ) correlations. The correlations were obtained by using the delta (baseline – postintervention) value of both groups pooled to increase the *n* and to evaluate the effect of doses of flavanones. When correlations were not significant, the *r* or ρ and *P* values are not given. CoQ, coenzyme Q; DBP, diastolic blood pressure; GPX, glutathione peroxidase; GR, glutathione reductase; HPJ, high-polyphenol-concentration orange juice; NPJ, normal-polyphenol-concentration orange juice; LPO, lipid peroxide; SBP, systolic blood pressure; SOD, superoxide dismutase; WC, waist circumference; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F2a; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

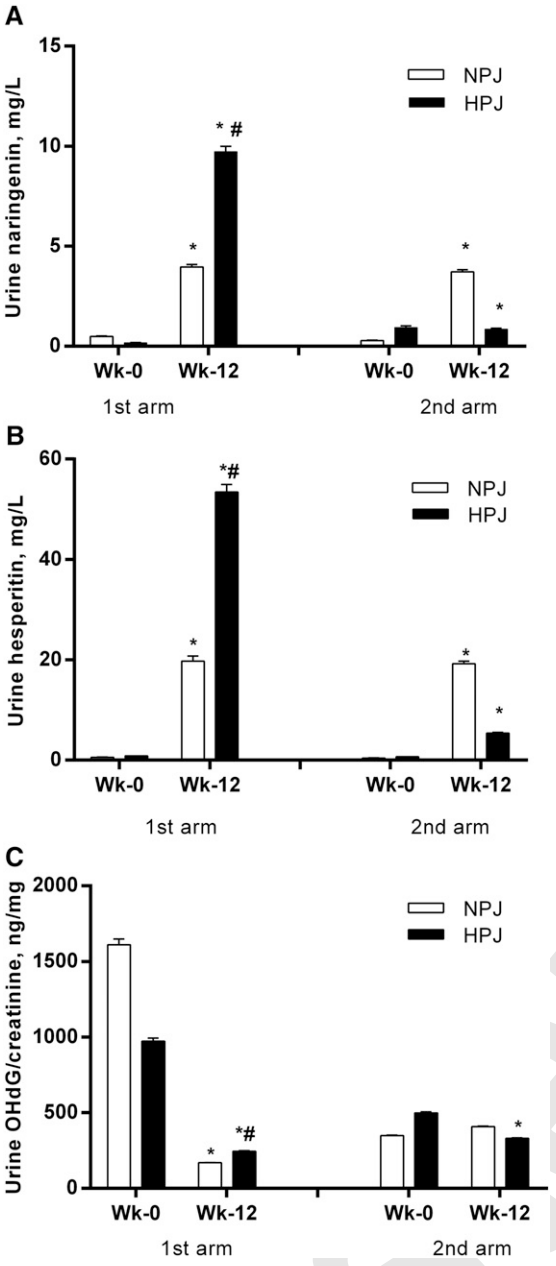

FIGURE 2 Urine concentrations of hesperidin (A) and narirutin (B) conjugates and 8-OHdG (C) in overweight and obese adults at baseline and after 12-wk HPJ and NPJ interventions. Values are means ± SEMs; $n = 46$ for NPJ and $n = 54$ for HPJ in the first arm and $n = 54$ for NPJ and $n = 46$ for HPJ during the second arm of the study. *Different from week 0 in each arm of the study, $P < 0.05$; #different from NPJ [AQ26] within each arm of the study, $P < 0.05$. HPJ, high-polyphenol-concentration orange juice; NPJ, normal-polyphenol-concentration orange juice; Wk, week; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

[AQ17] decrease lipid peroxidation (22). Moreover, Schär et al. (23) recently reported that flavanones derived from OJ did not improve cardiovascular disease biomarkers after 5 h in a well-designed acute intervention. Our data show that the consumption of both juices reduced urinary 8-iso-PGF_{2a}, which is recognized as one of the most reliable indices of lipid peroxidation because of its specificity and stability. These data agree with the results reported by Sánchez-Moreno et al. (24). However, we did not find a consistent effect on plasma lipid peroxidation biomarkers. In fact, neither oxLDL nor malondialdehyde was modified, and LPO was only reduced after the consumption of flavanones at 300 mg/d but not at 745 mg/d. To the best of our knowledge, there have not been any human intervention studies on the effects of flavanones on these biomarkers. In addition, another study observed dose-dependent pro-oxidant effects of naringenin and hesperidin (10), whereas Galati and O'Brien (25) identified naringenin but not hesperidin as inducing lipid peroxidation under the same conditions in which other flavanones exert antioxidant effects. Moreover, a recent review reported the pro-oxidant effects of high doses of flavonoids (9).

Several studies have established that supplementation with antioxidants appears to protect against DNA damage (13, 26, 27). As expected, we found that intervention with either of the experimental beverages produced a significant decrease in urinary 8-OHdG concentrations. Moreover, the observations that 8-OHdG was negatively correlated with CoQ_9 and CoQ_{10} and 8-iso-PGF_{2a} was negatively correlated with β-carotene and CoQ_9 reflect the importance of vitamin balance for the decrease in oxidative DNA damage. In agreement with our results, it is well accepted that the intake of exogenous antioxidants modifies the nonenzymatic antioxidant defense system (NEADS) plasma variables (28, 29) and specifically the intake of OJ, hesperidin, or narirutin may affect the NEADS (27, 30, 31). Naringenin acts by maintaining a vitamin E–sparing effect, which can lead to the neutralization of unsaturated membrane lipid peroxidation through its oxygen-scavenging effects (32, 33). Moreover, hesperidin antioxidant activity, which mostly involves scavenging hydroxyl radicals and superoxide, is more efficient when hesperidin is combined with vitamin C, a compound that is naturally present in OJ (34–37). Our data demonstrated differences in baseline plasma antioxidant molecules; however, after the interventions, the plasma NEADS variables improved independent of the type of OJ intake, reaching similar concentrations except for those of CoQ_9. Nonetheless, the relation between CoQ_9 and the other antioxidants confirms that it had a similar behavior pattern. This may indicate that, in our study, plasma antioxidant status was normalized after the nutritional intervention with exogenous antioxidants and did not differ between the doses of flavonoids that were ingested. However, we did not find any relation between CoQ_{10} and OJ intake that could explain the mechanism of its regulation. Nevertheless, CoQ_{10} is well known to be part of the NEADS in both plasma and cells and originates from endogenous synthesis and food intake.

A 12-wk intervention with either the NPJ or the HPJ induced a modification in the enzymatic antioxidant defense system. In particular, we observed a decrease in catalase activity, which was also reported by Jain et al. (38). This may be due to the scavenging activity of hesperidin, which reduces superoxide anions and consequently the LPO and hydrogen peroxide generated during normal cell metabolism, thereby reducing the need for catalase biosynthesis (37). This is supported by the fact that the intakes of hesperidin and narirutin were inversely correlated with catalase activity. In addition, lower concentrations of LPO and hydrogen peroxide, which are metabolized by GPX, may generate less GSSG (39); thus, the need for GR may have decreased, although this did not affect the glutathione-dependent antioxidant defense because the GSH and GSSG blood concentrations did not change after the interventions. In contrast, supplementation with OJ (i.e., hesperidin and narirutin) was found to increase SOD activity (33, 40, 41). Several studies in animals reported a relation between hesperidin and naringin and an increase in SOD gene expression (31, 40, 41);

this finding is in accordance with our findings after the HPJ intervention and provides a possible explanation for the improved antioxidant status observed after the supplementation. In fact, Cilla et al. (44) reported that the SOD induction observed after the consumption of a mixed-fruit beverage (grape-orange-apricot) may be more effective than the accumulation of exogenous antioxidants in the plasma. Notably, although GPX was not affected by OJ intake in the present study, we observed a relation between SOD and GPX, which may represent an increase in erythrocyte cell membrane antioxidant defense.

We also evaluated the influence of the OJ intake on metabolic syndrome clinical signs. The decrease in BMI, WC, and plasma leptin after the intake of either of the juices was due to the decrease in energy intake and was not associated with the flavanone supplementation. In fact, BMI, WC, and plasma leptin were correlated with energy intake but not with urinary flavanones. To the best of our knowledge, there is no evidence that associates weight loss with flavanones (45). However, the subjects in our study showed a significant decrease in DBP and SBP after both of the 12-wk interventions, as previously reported (30). Furthermore, the inverse correlations between the flavanones and SBP and DBP support this hypotensive effect, which has been attributed to an NO-mediated vasodilatation mechanism (46).

Our intervention caused an increase in basal glucose concentrations that may have been associated with either the daily addition of 500 mL dietary OJ or the decrease in insulin secretion. Nevertheless, the glucose and insulin concentrations at the beginning and at the end of the interventions were within normal ranges. In addition, the HOMA-IR index, which estimates insulin sensitivity, was not modified after the intervention with either of the OJs. Other studies have attributed antidiabetogenic properties to hesperidin and naringin administered alone to rats (33). Therefore, more studies are needed to elucidate the effects of different doses of polyphenols on blood glucose and insulin secretion and whether the food matrix may modulate these effects.

Finally, in our study, supplementation with hesperidin and narirutin from OJ did not demonstrate significant changes in lipid metabolism. This finding is in agreement with Demonty and Lin (47), who found that a 4-wk intervention with either 500 mg/d pure naringin or 800 mg/d pure hesperidin did not have an effect on lipid metabolism in 204 moderately hyperlipidemic subjects. In contrast, other trials have shown that hesperidin at doses >400 mg/d (administered either in OJ or as a pure compound) might improve the blood lipid profile (48, 49). Note that, in our study, the concentrations of blood lipids including TGs and LDL cholesterol were low compared with those observed in these previous studies. However, as reported in other studies (3, 50, 51), the NPJ intervention in our study showed a decrease in apoB concentration, which is a major component of LDL cholesterol.

In conclusion, our results show that the consumption of OJ with at least 300 mg flavanones over a 12-wk period enhanced the antioxidant defense system, protected against DNA damage and lipid peroxidation, and improved BP in overweight and obese adults. The elucidation of the specific role of each flavanone and their mechanisms of action will require further study.

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Review

A Systematic Review of the Efficacy of Bioactive Compounds in Cardiovascular Disease: Phenolic Compounds

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Abstract: The prevalence of cardiovascular diseases (CVD) is rising and is the prime cause of death in all developed countries. Bioactive compounds (BAC) can have a role in CVD prevention and treatment. The aim of this work was to examine the scientific evidence supporting phenolic BAC efficacy in CVD prevention and treatment by a systematic review. Databases utilized were Medline, LILACS and EMBASE, and all randomized controlled trials (RCTs) with prospective, parallel or crossover designs in humans in which the effects of BAC were compared with that of placebo/control were included. Vascular homeostasis, blood pressure, endothelial function, oxidative stress and inflammatory biomarkers were considered as primary outcomes. Cohort, ecological or case-control studies were not included. We selected 72 articles and verified their quality based on the Scottish Intercollegiate Guidelines Network, establishing diverse quality levels of scientific evidence according to two features: the design and bias risk of a study. Moreover, a grade of recommendation was included, depending on evidence strength of antecedents. Evidence shows that certain polyphenols, such as flavonols can be helpful in decreasing CVD risk factors. However, further rigorous evidence is necessary to support the BAC effect on CVD prevention and treatment.

Keywords: bioactive food compounds; cardiovascular diseases; polyphenols; phenols; flavonols

1. Introduction

The prevalence of cardiovascular disease (CVD) is rising and is the prime cause of death in all developed countries [1], and one of the most important health issues in developing countries [2]. While some risk factors cannot be changed, such as family history, ethnicity and age, detection and control of modifiable factors such as, blood pressure (BP), high cholesterol, obesity, type 2 diabetes (T2D) or unhealthy diets can help to prevent intermediate risk CVD processes like inflammation or oxidative stress. Thus, primary prevention of CVD by identifying and treating at-risk individuals remains a major public-health priority. A healthy life style is the main pre-emptive approach [3,4].

Dietary habits are quite different around world; nevertheless, certain consumption patterns are common worldwide, inclusion of fruits and vegetables or products like cocoa, coffee or condiments is a merging point. Bioactive compounds (BAC) are "extra nutritional" constituents that are present in small quantities in plant products and lipid rich foods [5]. The growing body of scientific evidence indicates that certain BAC play a beneficial role in CVD prevention [6–11]. BAC oral supplements along with a usual diet can increase the intake of ingredients reputed to have clinical benefits. These supplements are, usually, an addition to the healthy diet, and not as a conventional food or the sole item of a meal [12].

Putative beneficial biological effects such as antilipidemic, antihypertensive, anti-glycaemic, antithrombotic and anti-atherogenic effects are attributed to BAC. In the present study, the main goal was to examine the scientific evidence of BAC in the prevention and treatment of CVD by a systematic review of randomized clinical trials (RCTs). The BAC considered in this review were all those related to the phenolic compounds.

Phenolic compounds such as stilbenes like the resveratrol (3,5,4′-trihydroxystilbene) can be found principally in the skin of grapes and are produced in other plants, such as peanuts [6]. Red wine is a rich source of resveratrol and is thought to confer the cardio protective effects associated with moderate consumption of wine [13]. Within the catechols family, curcuminoids are multifunctional natural compounds found in native Indonesian plants, with promising cardio protective and anti-inflammatory properties and mainly present in the dried rhizomes of *Curcuma longa L*. (commonly known as turmeric) [14].

In relation to polyphenols, there are six basic subclasses of flavonoids: flavones, anthocyanins, flavanones, flavonols, isoflavones, and the flavanols, including the flavanol oligomers, the proanthocyanidins that are further subdivided into 16 species including the procyanidins, oligomers of the flavan-3-ols catechin and epicatechin, and the prodelphinidins, oligomers of the gallocatechins [15]. In this review, we utilized equations to divide the results according to the most relevant classes.

Specifically, we examined the effects of BAC on BP, lipid profile [triacylglycerol (TAG), cholesterol, high and low density lipoproteins(HDL and LDL)], carbohydrate (CHO) metabolism (glucose, insulin, and insulin resistance (IR)), oxidative stress, inflammation and endothelial function (EF). Furthermore, we gave a recommendation for consumption based on the evidence grade according to Scottish Intercollegiate Guidelines Network (SIGN) [16].

2. Methodology

We developed a literature search in Medline by PubMed (U.S. National Library of Medicine and the NIH), and in LILACS and EMBASE, including publications in English, Spanish and Portuguese until December 2014. Studies eligible for this review included: randomized controlled trials (RCTs) in healthy and unhealthy adults, with prospective, parallel or crossover designs, with full text, and whose primary outcomes were vascular homeostasis, BP, oxidative stress and/or inflammatory biomarkers; we excluded those studies with cohort, ecological or case-control design, those which analysed a drug, or when BAC were combined with other compounds. However, there was no restriction on publication type or sample size.

2.1. Search Equation

Due to the diversity of the chemical structures of phenol compounds (Figure 1), the type of BAC can present different effects in CVD; consequently, we evaluated the more relevant groups. We included different keywords in the search equation of bioactive compounds, including: Phenols (*stilbenes*, *catechols*, *flavonoids*, *anthocyanins*, *flavanones*, *isoflavones*, *polyphenols*, *phenolic acids*, *gallic acid and hydroquinones*). We combined the MeSH term "cardiovascular diseases" with each bioactive compound as MeSH Major Topic, together with NOT "review" (Publication Type) in PubMed. However, equations in the Spanish language were used when the search was carried out in LILACS *i.e.*, (tw:(polifenoles)) AND (tw:(enfermedad cardiovascular)) AND NOT (tw:(revisión)). When consulting the database EMBASE, the equations were elaborated as "catechols"/mj AND "cardiovascular diseases"/mj "NOT review". Articles published before 1990 were discarded because they did not comply the inclusion criteria stablished.

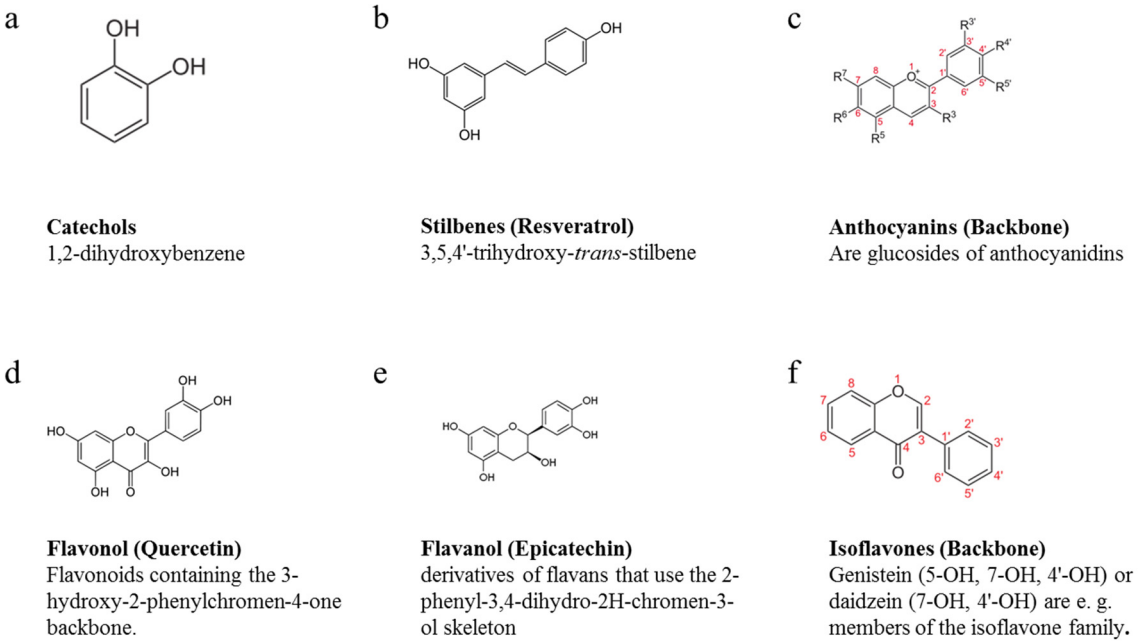

Figure 1. Chemical diversity polyphenols. Simple phenols are represented by (**a**) catechols and (**b**) stilbenes, and polyphenols in (**c**) anthocyanins, (**d**) flavonols, (**e**) flavanols and (**f**) isoflavones.

After the review process by proofreading staff, we included four additional articles. Three of them were not located by our search criteria; the other appeared in our initial search, but its main outcome in relation to exercise did not comply with the requirement for inclusion in the review. Nevertheless, after a second approach by the proofreading staff, we also decided to include it (see footnotes in the Tables 1–3).

2.2. Selection and Evaluation

First, both titles and abstracts were identified independently by two reviewers, for exclusion of those articles that did not fit with the language, date, subject matter, design and outcomes established. Then, full-text publications were classified by pathologies according to outcomes analysed in each study.

Moreover, RCTs were finally selected if they obtained a score between 3 and 5 according to the Jadadscale [17]. This method attempts to reduce bias for RCTs, ensuring a certain quality in the evidence; it took into account if they were randomized, blinded and provided detailed information about patients.

Furthermore, we verified the quality of selected articles by the Scottish Intercollegiate Guidelines Network (SIGN) [16]. Diverse quality levels of scientific evidence are established according to two features: the design and bias risk of a study. The levels are from 1++, when the information is considered as high quality, to 4 when the information is considered as very low quality. Signs are used to reporting with reference to compliance degree of key criteria associated with potential bias (1++, 1+, 1-, 2++, 2+, 2-, 3, and 4). Additionally, we included a grade of recommendation, based on the evidence strength of the antecedents, whose levels are A, B, C, D, with "A" being highly recommended and "D" not recommended. These grades of recommendation by SIGN guidelines are equivalent to those designated by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), as evidence criteria: convincing, probable, possible and insufficient [18].

3. Results and Discussion

In total, 831 RCT's were found using the equations proposed in the different databases (EMBASE, LILACS and PubMed). We excluded 717 due to obvious irrelevance, leaving 114 papers in full to read (Figure 2). After papers were read and evaluated using the Jadad scale, 76 articles were selected for the final review, and are included in Tables 1–3.

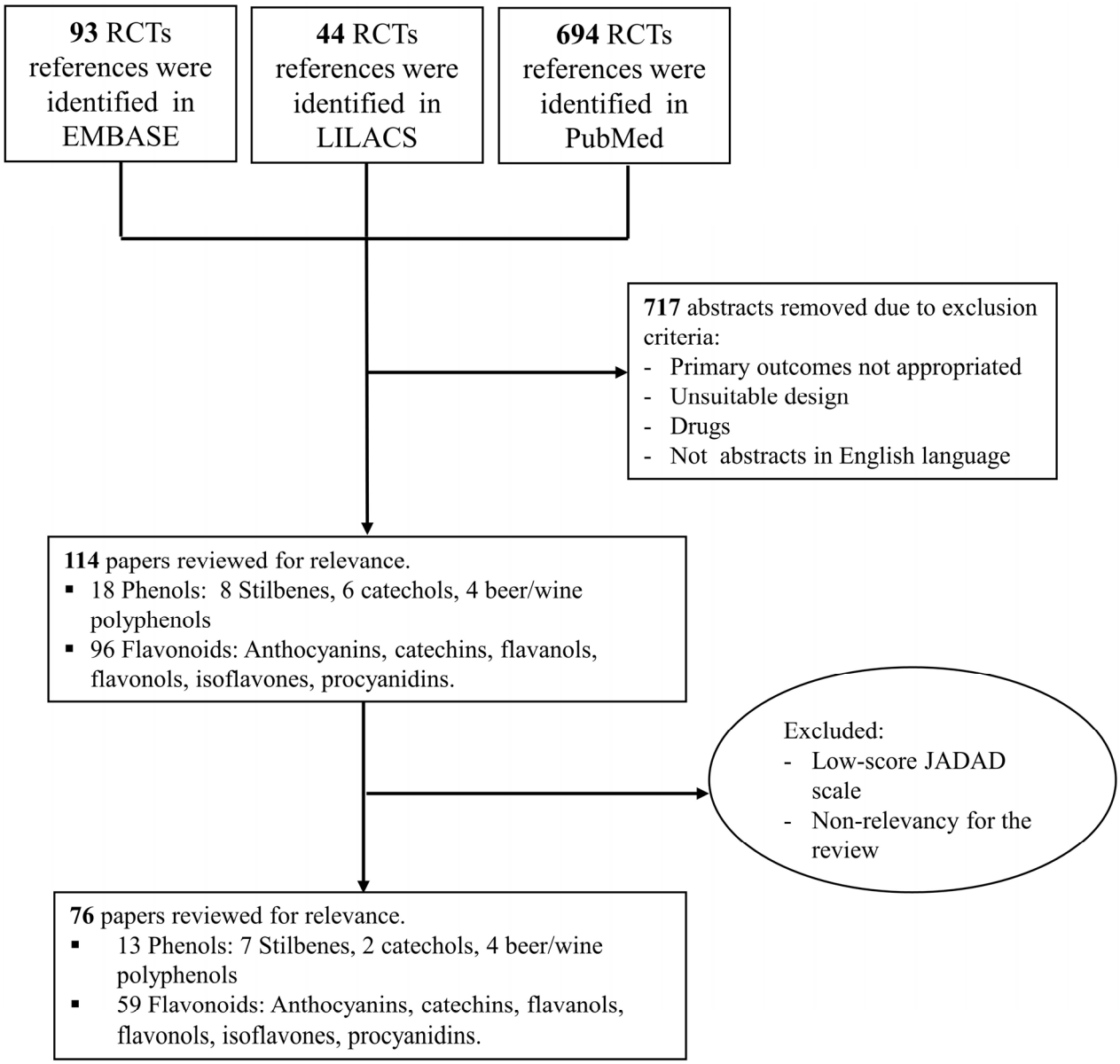

Figure 2. Review Flow Diagram.

Table 1. RCTs of phenolic compounds (catechols, stilbenes and beer/wine) in CVD risk.

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Phenols (Stilbenes)	Wong *et al.* (2011) [19]	5	2B, X (1 h)	(19) Overweight/obese + ↑ BP men or post-menopausal women	30 mg, 90 mg, 270 mg RSV *vs.* PCB	EF	↑ EF, more with highest dose
Phenols (Stilbenes)	Wong *et al.* (2013) [20] *	5	2B, X (1 h)	(28) obese subjects	Acute intervention: 75 mg/ trans-resveratrol (Resvida) *vs.* PCB after chronic intervention	FMD	↑ FMD
Phenols (Stilbenes)	Wong *et al.* (2013) [20] *	5	2B, X (6 weeks)	(28) obese subjects	75 mg/day trans-resveratrol (Resvida) *vs.* PCB	BP, AR, BMI, FMD	↑ FMD
Phenols (Stilbenes)	Bo *et al.* (2013) [21]	5	2B, X (60 days(wash-out 30 days))	(50) Healthy smokers	500 mg RSV/d *vs.* PCB	BP, Anthropometry, lipids profile, CHO metabolism, TAS, hsCRP,	↓ hsCRP, TAG, ↑ TAS
Phenols (Stilbenes)	Militaru *et al.* (2013) [22]	3	2B, Ctrl, PA (60 days)	(166) BMI 24–27 kg/m^2, stable angina pectoris	20 mg/day RSV, 20 mg/day RSV + 112 mg/day CF, 112 mg/day CF	Lipids profile, hsCRP, left ventricular function markers	↓ TC, TAG greater in RSV, hsCRP greater in CF, NT-proBNP more effective RSV+CF
Phenols (Stilbenes)	Tomé-Carneiro *et al.* (2013) [23]	4	3B, PCB (1 year)	(75) Stable CAD patients	350 mg/day GE, 350 mg/day GE-RES *vs.* PCB (6 months); double dose next 6 months	PBMCs, inflammatory and fibrinolytic biomarkers	↑ adiponectin, ↓ PAI-1, significantly activated or inhibited 6 key inflammation-related transcription factors in PBMCs
Phenols (Stilbenes)	Tomé-Carneiro *et al.* (2012) [24]	4	3B, PCB (6 months)	(75) Primary prevention of CVD	350 mg/day GE, 350 mg/day GE-RES *vs.* PCB	Lipids profile, oxidized LDL	↓ LDLc, ApoB, LDLox and LDLox/ApoB ratio, ↑ nonHDLc/ApoB ratio in GE-RES

Table 1. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Phenols (Stilbenes)	Tomé-Carneiro *et al.* (2013) [25]	5	3B, PCB, dose–response (1 year)	(35) T2D, HT with CAD	350 mg/day GE, 350 mg/day GE-RES *vs.* PCB (6 months); double dose next 6 months	PBMCs, inflammatory, fibrinolytic biomarkers	↓ CCL3, IL-1β, TNF-α expression, ↑ transcriptional repressor LRRFIP-1 in PBMCs with GE-RES
Phenols (Stilbenes)	Tomé-Carneiro *et al.* (2012) [26]	4	3B, PCB (1 year)	(75) Primary prevention of CVD	350 mg/day GE, 350 mg/day GE-RES *vs.* PCB (6 months); double dose next 6 months	Inflammatory and fibrinolytic biomarkers	↓ CRP, TNF-α, PAI-1, IL-6/IL-10 ratio, sICAM ↑ IL-10, adiponectin in GE-RES
Phenols (Catechols)	Alwi *et al.* (2008) [27]	4	2B, PCB (2 months)	(75) ACS patients	45 mg/day, 90 mg/day or 180 mg/day curcumin *vs.* PCB	Lipids profile	Not significant effect
Phenols (Catechols)	Chuengsamarn *et al.* (2014) [28]	5	2B, PCB (6 months)	(240) T2D patients	750 mg/day curcumin *vs.* PCB	BP, anthropometry, lipids profile, adiponectin, leptin, CHO metabolism, PWV, uric acid	↓ PWV, HOMA, TAG, uric acid, abdominal obesity and leptin, ↑ adiponectin.
Polyphenols (Wine/beer)	Botden *et al.* (2012) [29]	4	2B, PCB, three-period X (4 weeks)	(61) HT subjects	280 mg/day red wine polyphenols or 560 mg/day red wine polyphenols *vs.* PCB	BP	No significant effect

Table 1. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Polyphenols (Wine/beer)	Chiva-Blanch *et al.* (2014) [30]	4	2B, PCB, X (4 weeks)	(36) High risk of CVD males	Beer (30 g alcohol/day), the equivalent amount of polyphenols in the form of non-alcoholic beer, or gin (30 g alcohol/day)	Circulating endothelial progenitor cells and EPC-mobilizing factors	Beer and non-alcoholic beer interventions, ↑-circulating EPC. No significant differences were observed after the gin period
Polyphenols (Wine/beer)	Chiva-Blanch *et al.* (2012) [31]	3	X (4 weeks)	(67) High risk of CVD males	Red wine (30 g alcohol/day), the equivalent amount of dealcoholized red wine, or gin (30 g alcohol/day)	BP and plasma nitric oxide	Dealcoholized red wine ↓ DBP and SBP
Polyphenols (Wine/beer)	Chiva-Blanch *et al.* (2012) [32]	3	X (4 weeks)	(67) High risk of CVD males	Red wine (30 g alcohol/day), the equivalent amount of dealcoholized red wine, or gin (30 g alcohol/day)	Inflammatory biomarkers	Alcohol ↑ IL-10 and ↓ macrophage-derived chemokine concentrations. Phenolic compounds of Red wine ↓ serum concentrations of ICAM-1, E-selectin, and IL-6

2B, double-blinded, 3B, triple-blinded, ACS, acute coronary syndrome; Apo, apolipoprotein; BMI, body mass index; BP, blood pressure; CAD, chronic artery disease; CHO, carbohydrate; CCL-3, chemokine (C–C motif) ligand 3 CRP, C-reactive protein; hsCRP, high sensitivity c-reactive protein; Ctrl, control, CVD, cardiovascular disease; EF, endothelial function; EPC, endothelial progenitor cells; FMD, flow mediated dilation; GE, grape extract; GE-RES, grape extract containing RSV (8mg); HDLc, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; HT, hypertension; sICAM, soluble intercellular adhesion molecule; IL, interleukin; LDLc, low-density lipoprotein cholesterol; LDLox, oxidized LDL; LRRFIP-1, leucine rich repeat (in FLII) interacting protein 1; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; PAI-1, plasminogen activator inhibitor-1; PCB, placebo, PBMCs, peripheral blood mononuclear cells; PWV, pulse wave velocity; RSV, resveratrol; TAG, triacylglycerols; TC, total cholesterol; TNF-α,tumour necrosis factor alpha; T2D, type 2 diabetes; X, crossover design. * Included after proofreading.

Table 2. RCTs of polyphenols (anthocyanins, catechins, flavanols and flavonols) in CVD risk.

Group (Class)	Author/ Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Anthocyanins)	Kuntz (2014) [33]	4	2B, PCB, X (14 days)	(30) Healthy females	330 mL/day beverages (PCB, juice or smoothie with 8.9, 983.7 and 840.9 mg/L ACN, respectively)	Inflammatory and oxidative stress biomarkers	↑ SOD and CAT after ACN. ↓ MDA after ACN ingestion.
Flavonoids (Anthocyanins)	Curtis *et al.* (2009) [34]	5	PCB, PA (12 weeks)	(57) Postmenopausal women	500 mg/day ACN *vs.* PCB	BP, CHO metabolism, lipids profile, inflammatory biomarkers, platelet reactivity	No significant effect
Flavonoids (Anthocyanins)	Hassellund *et al.* (2013) [35]	5	2B, PCB, X (4 weeks)	(31) Pre-hypertensive males	640 mg/day ACN *vs.* PCB	Lipids profile, CHO metabolism, inflammatory and oxidative stress biomarkers	↑ HDLc and glucose after anthocyanin *versus* PCB treatment. No effects were observed on inflammation or oxidative stress *in vivo*, except for vWf
Flavonoids (Anthocyanins)	Dohadwala *et al.* (2011) [36]	4	Open-label, (2 and 4 hour acute study)	(15) CAD subjects	835 mg total polyphenols, 94 mg anthocyanins *vs.* PCB	Vascular function	No significant effect
Flavonoids (Anthocyanins)	Dohadwala *et al.* (2011) [36]	4	X, 2B, PCB (4 weeks, 2 week washout)	(44) CAD subjects	835 mg total polyphenols, 94 mg anthocyanins *vs.* PCB	Vascular function	↓ Carotid femoral pulse wave activity
Flavonoids (Catechins)	Miyazaki *et al.* (2013) [37]	4	2B, PCB (14 weeks)	(52) Healthy subjects	630.9 mg/day Green Tea Catechins *vs.* Ctrl	CVD risk markers	No significant effect
Flavonoids (Catechins)	de Maat *et al.* (2000) [38]	3	1B, PCB, PA (4 weeks)	(64) Healthy subjects	Black tea (3 g/day), green tea (3 g/day), green tea polyphenol isolate capsules (3.6 mg/day) and mineral water.	Inflammatory and endothelial markers	Negative correlation between the levels of the antioxidant β-carotene and the inflammation markers IL6 and fibrinogen

Table 2. *Cont.*

Group (Class)	Author/ Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Catechins)	Widmer *et al.* (2013) [39]	3	2B, Ctrl (4 months)	(52) Early atherosclerosis	30 mL/day simple Olive Oil *vs.* 30 mL/day of EGCG-supplemented Olive Oil	EF, inflammation and oxidative stress	Only significant when merging data of both groups the EF was improved.
Flavonoids (Catechins)	Nagao *et al.* (2007) [40]	4	2B, PA (12 weeks)	(240) Visceral fat-type obesity	Green tea containing 583 mg/day catechins (catechin group) *vs.* 96 mg/day catechins (Ctrl group)	Anthropometric measurements, body fat composition and CVD risk	↓ body weight, BMI, body fat ratio, body fat mass, waist circumference, hip circumference, visceral fat area, and subcutaneous fat area, SBP, LDLc
Flavonoids (Flavanols)	Farouque *et al.* (2006) [41]	5	2B, PCB (6 weeks)	(40) Healthy males	Flavanol-rich chocolate bar and cocoa beverage (total flavanols, 444 mg/day) *vs.* matching isocaloric PCBs (total flavanols, 19.6 mg/day)	EF and adhesion molecules	No significant effect
Flavonoids (Flavanols)	Berry *et al.* (2010) [42] *	4	2B, X (2 h, 3–7 days washout)	(21) overweight/obese subjects	HF, 701 mg or LF, 22 mg cocoa	BP, HR, FMD	↑ DBP after exercise were attenuate by HF, improvement of FMD with HF
Flavonoids (Flavanols)	Davison *et al.* 2008 [43] *	4	2B, PCB, PA (12 weeks)	(98) overweight/obese subjects	902 mg cocoa flavanols/day *vs.* 36 mg cocoa flavanols/day With/without exercise protocol	BP, HDLc, LDLc, TG, HOMA, FMD	↑ FMD at 6 and 12 weeks with HF *vs.* LF, ↑ DBP, BP mean, improvement in HOMA (independent of exercise)

Table 2. *Cont.*

Group (Class)	Author/ Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Flavanols)	West *et al.* (2014) [44]	3	2B, PCB, X (4 weeks, 2 weeks washout)	(30) Middle-aged overweight	37 g/day of dark chocolate and a sugar-free cocoa beverage (total flavanols = 814 mg/day) *vs.* low-flavonol chocolate and cocoa free beverage (total flavanols = 3 mg/day)	EF, BP	↑ Basal and peak diameter of the brachial artery and basal blood flow volume.
Flavonoids (Flavanols)	Faridi *et al.* (2008) [45]	4	X, Ctrl, 1B (1 days, 7 days washout)	(45) Overweight subjects	Solid dark chocolate bar (821 mg flavanols) *vs.* cocoa-free PCB bar (0 mg flavanols)	EF, BP	Solid dark chocolate improved EF; also ↓ BP
Flavonoids (Flavanols)	Faridi *et al.* (2008) [45]	4	X, Ctrl, 1B (1 days, 7 days washout)	(44) Overweight subjects	Sugar-free cocoa (805.2 mg flavanols), sugared cocoa (805.2 mg flavanols), *vs.* PCB (0 mg flavanols).	EF, BP	Liquid cocoa ingestion improved EF; sugar-free cocoa ↓ BP
Flavonoids (Flavanols)	Davison *et al.* (2010) [46]	3	2B, PA (6 weeks)	(52) Men and postmenopausal women with untreated mild HT	33, 372, 712 or 1052 mg/day of cocoa flavanols	24-h BP	No significant effect
Flavonoids (Flavanols)	Grassi *et al.* (2008) [47]	3	X, Ctrl, 1B (15 days)	(19) HT with Impaired glucose tolerance	Flavonol-rich dark chocolate (110.9 mg epicatechin, 36.12 mg catechin, 2.5 mg quercetin, 0.03 mg kaempferol, and 0.2 mg isorhamnetin)/d or flavonol-free white chocolate (0.04 mg/day catechins)	EF, IR, β-cell function, BP, CRP, TC	↓ IR, BP, TC, LDLc. ↑ insulin sensitivity, EF

Table 2. *Cont.*

Group (Class)	Author/ Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Flavanols)	Flammer *et al.* (2012) [48]	3	2B, PCB (2 hours)	(20) CHF patients	40 g Flavonol rich chocolate (624 mg total flavanols) *vs.* 28.4 g Ctrl chocolate (0 mg flavanols	EF and platelet function in the short term	Improvement of vascular function in patients with CHF
Flavonoids (Flavanols)	Flammer *et al.* (2012) [48]	3	2B, PCB (2 and 4 weeks)	(20) CHF patients	40 g/day Flavonol rich chocolate (624 mg total flavanols) *vs.* 28.4 g/day Ctrl chocolate (0 mg flavanols)	EF and platelet function in long term by FMD	Improvement of vascular function in patients with CHF
Flavonoids (Flavanols)	Heiss *et al.* (2010) [49]	3	Ctrl, 2B, X (30 days)	(16) CAD patients	High-flavanol intervention (375 mg/day) and a macronutrient- and micronutrient-matched low-flavanol intervention (9 mg/day) twice daily	EF and enhancement and function of circulating angiogenic cells	↑ EF, CD34+/KDR+- Circulating angiogenic cells. ↓ SBP
Flavonoids (Flavanols)	Horn *et al.* (2013) [50]	3	2B, X (30 days)	(16) CAD patients	High-flavanol intervention (375 mg/day) and a macronutrient- and micronutrient-matched low-flavanol intervention (9 mg/day) twice daily	Circulating endothelial micro particles, markers of endothelial integrity, EF	↑ Endothelial micro-particles and EF. Improvement of endothelial integrity
Flavonoids (Flavanols)	Balzer *et al.* (2008) [51]	5	2B, PCB, three-period X (2 h)	(10) Diabetic subjects	Single-dose ingestion of cocoa, containing increasing concentrations of flavanols (75, 371, and 963 mg)	EF	Single ingestion of flavanol-containing cocoa was dose-dependently acute increases in circulating flavanols and EF
Flavonoids (Flavanols)	Balzer *et al.* (2008) [51]	5	2B, PCB, PA (30 days)	(41) Diabetic subjects	963 mg/day Flavanol-rich cocoa *vs.* nutrient-matched Ctrl (75 mg/day flavanols)	EF	Flavanol-containing cocoa ↑ baseline EF

Table 2. *Cont.*

Group (Class)	Author/ Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Flavonols)	Larson *et al.* (2012) [52]	3	2B, PCB, X (1 days, 7 days washout)	(5) Healthy males	1095 mg quercetin aglycone *vs.* PCB	Angiotensin-converting enzyme, endothelin-1, BP	No significant effect
Flavonoids (Flavonols)	Conquer *et al.* (1998) [53]	3	2B (28 days)	(27) Healthy subjects	4 capsules (1.0 g quercetin/day) *vs.* rice flour PCB	BP, lipids profile, thrombogenic risk factors	No significant effect
Flavonoids (Flavonols)	Suomela *et al.* (2006) [54]	3	2B, PCB, X (4 weeks, 4 weeks washout)	(14) Healthy males	Oat meal with 78 mg/day flavonol aglycones (sea buckthorn) *vs.* Ctrl	CVD risk markers	No significant effect
Flavonoids (Flavonols)	Edwards *et al.* (2007) [55]	3	2B, PCB, X (28 days)	(41) Prehypertension and hypertension	730 mg quercetin/day *vs.* PCB	BP, oxidative stress	↓ BP in hypertensive group
Flavonoids (Flavonols)	Larson *et al.* (2012) [52]	3	2B, PCB, X (1 days, 2 days washout)	(12) HT stage 1 males	1095 mg quercetin aglycone *vs.* PCB	Angiotensin-converting enzyme, endothelin-1, BP	↓ BP in Hypertensive men

8-iso-PGF2α, 8-iso-prostaglandin F2α; 1B, one-blind, 2B, double-blinded; ACN, anthocyanins; Apo, apolipoprotein; BMI, body mass index; BP, blood pressure; CAD, chronic artery disease; CAT, catalase; CHF, chronic heart failure; CHO, carbohydrate; CRP, C-reactive protein; hsCRP, high sensitivity c-reactive protein; Ctrl, control, CVD, cardiovascular disease; DXA, Dual-energy X-ray absorptiometry; EF, endothelial function; EGCG, epigallocatechin gallate; ESRD, European and North American end-stage renal disease; FM, fat mass; FFM, fat-free mass; FMD, flow mediated dilation; HDLc, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; HR, heart rate; HT, hypertension; sICAM, soluble intercellular adhesion molecule; IGF-1, insulin-like growth factor-1; IR, insulin resistance; IL, interleukin; LDLc, low-density lipoprotein cholesterol; MDA, malonaldehyde; MPFF, micronized purified flavonoid fraction; MPI, milk protein isolate; NTG, nitro-glycerine-mediated dilation; PA, parallel design, PAI-1, plasminogen activator inhibitor-1; PCB, placebo, PBMCs, peripheral blood mononuclear cells; PS, plant sterols; PWV, pulse wave velocity; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; SOD, superoxide dismutase; TC, total cholesterol; TGF, transforming growth factor; T2D, type 2 diabetes; VCAM, soluble vascular cellular adhesion molecule; vWf, von Willebrand factor; X, crossover design. * Included after proofreading.

Table 3. RCTs of polyphenols (isoflavones and procyanidins) in CVD risk.

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Isoflavones)	McVeigh *et al.* (2006) [56]	3	1B, X (57 days, 4 weeks washout)	(35) Healthy males	Milk protein isolate (MPI), low-isoflavone soy protein isolate (low-iso SPI; 1.64 ± 0.19 mg aglycone isoflavones/day), and high-isoflavone SPI (high-iso SPI; 61.7 ± 7.4 mg aglycone isoflavones/day)	Lipids profile	↓ TC/HDLc, LDLc/HDLc, and Apo B/Apo A-I with both SPI treatments than with MPI treatment
Flavonoids (Isoflavones)	Sanders *et al.* (2002) [57]	3	X (17 days, 25 days washout)	(22) Healthy subjects	56 *vs.* 2 mg isoflavones/day	Lipids profile, fibrinogen, and active TGF-β, factor VII coagulant and PAI-1	↑ HDL and Apo A1 in high-isoflavone
Flavonoids (Isoflavones)	Thorp *et al.* (2008) [58] *	5	2B, PCB, X (6 weeks)	(91) Hypercholesterolemia	24 g SP+70–80 mg ISOs (diet S) *vs.* 12 g SP + 12 g dairy protein (DP) + 70–80 mg ISOs (diet SD) *vs.* 24 g DP without ISOs (diet D)	HDLc, LDLc, TC	No significant effect
Flavonoids (Isoflavones)	Atkinson *et al.* (2004) [59]	5	2B, PCB (12 months)	(205) Female	43.5 mg red clover-derived isoflavones/day *vs.* PCB	Lipids profile, BP, fibrinogen and PAI-1	No significant effect
Flavonoids (Isoflavones)	Marini *et al.* (2010) [60]	5	2B,PCB (24 months)	(138) Females with low bone mass	54 mg/day genistein aglycone *vs.* PCB	Lipids profile, CHO metabolism, HOMA, fibrinogen, osteoprotegerin and homocysteine	↓ fasting glucose and insulin, HOMA, fibrinogen and homocysteine
Flavonoids (Isoflavones)	Hodis *et al.* (2011) [61]	5	2B, PCB (2 years)	(350) Postmenopausal women	25 g/day soy protein (91 mg/day aglycone isoflavone equivalents) *vs.* PCB	Atherosclerosis progression	No significant effect

Table 3. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Isoflavones)	Atteritano *et al.* (2007) [62]	5	2B, PCB (24 months)	(191) Postmenopausal women	54 mg/day genistein *vs.* PCB	Lipids profile, CHO metabolism, HOMA, fibrinogen, sVCAM-1, sICAM-1, 8-iso-PGF2α, and osteoprotegerin	↓ Fasting glucose and insulin as well as HOMA, fibrinogen, 8-iso-PGF2α, sICAM-1, and sVCAM-1. ↑ Serum osteoprotegerin
Flavonoids (Isoflavones)	Garrido *et al.* (2006) [63]	3	PCB (12 weeks)	(29) Postmenopausal women	100 mg/day isoflavones *vs.* PCB	Lipids profile, CHO metabolism and platelet thromboxane A2 receptor density. BP, BMI, subcutaneous fat	↓ Thromboxane A2 after the experimental treatment.
Flavonoids (Isoflavones)	Hall *et al.* (2005) [64]	4	2B, PCB, X (8 weeks, 8 weeks washout)	(117) Postmenopausal women	Isoflavone-enriched (genistein-to-daidzein ratio of 2:1; 50 mg/day) *vs.* PCB cereal	Inflammatory and vascular homeostasis biomarkers	↓ CRP
Flavonoids (Isoflavones)	Rios *et al.* (2008) [65]	3	2B, PCB (6 months)	(47) Postmenopausal women	40 mg/day isoflavone *vs.* casein PCB	Lipids profile	No significant effect
Flavonoids (Isoflavones)	Villa *et al.* (2009) [66]	3	PCB (24 weeks)	(50) Postmenopausal women	54 mg/day genistein *vs.* PCB	Anthropometric measures, lipid profile, CHO metabolism and C-peptide evaluation, IR and EF	HOMA and fasting glucose levels significantly improved
Flavonoids (Isoflavones)	Liu *et al.* (2012) [67]	4	2B, PCB (6 months)	(180) Postmenopausal women	15 g/day soy protein and 100 mg/day isoflavone (Soy group), *vs.* 15 g/day milk protein and 100 mg/day isoflavone (Iso group) *vs.* 15 g/day milk protein (PCB)	Lipids profile, inflammatory markers and composite cardiovascular	No significant effect

Table 3. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Isoflavones)	Yang *et al.* (2012) [68]	3	Open-labelled, prospective (24 week)	(130) Healthy Taiwanese postmenopausal women	35 mg/day *vs.* 70 mg/day soy extract[a]	Lipids profile	↓ TC, LDLc in patients with TC >200 mg/dL
Flavonoids (Isoflavones)	Liu *et al.* (2013) [67]	5	2B, PCB (6 months)	(270) Pre-hypertensive women	40 g/day soy flour (whole soy group), 40 g/day low-fat milk powder + 63 mg/day daidzein (daidzein group), *vs.* 40 g/day low-fat milk powder (PCB)	Anthropometric indicators and body composition	No significant effect
Flavonoids (Isoflavones)	Aubertin-Leheudre *et al.* (2008) [69]	3	2B, PCB (6 months)	(50) Obese postmenopausal women	70 mg/day isoflavones *vs.* PCB	Body composition (DXA), and Lipid profile and CHO metabolism	No significant effect
Flavonoids (Isoflavones)	Choquette *et al.* (2011) [70]	4	2B, PCB (6 months)	(100) Overweight to obese postmenopausal women	PCB or isoflavones (70 mg/day) or exercise + PCB or exercise + isoflavones (70 mg/day). Exercise consisted of three weekly sessions of resistance training and aerobics	Body composition, lipids profile, CHO metabolism and HOMA.	No significant effect
Flavonoids (Isoflavones)	Aubertin-Leheudre M *et al.* (2007) [71]	3	2B,PCB (12 months)	(56) Obese postmenopausal women	70 mg/day isoflavones[b] (+weight loss exercise program from the 6 months) *vs.* PCB	Anthropometry, lipids profile, CHO metabolism, CRP	↓ body weight, BMI, total and abdominal FM (kg and %), ↑ FFM/FM ratio with exercise program
Flavonoids (Isoflavones)	Hodgson *et al.* (1999) [72]	3	2B, PCB, PA (8 weeks)	(59) High-normal BP	55 mg/day isoflavonoid *vs.* PCB	8-iso-PGF2α	No significant effect

Table 3. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Isoflavones)	Sagara *et al.* (2004) [73]	3	PCB, 2B, PA (5 weeks)	(61) Men with relatively higher BP or TC	Diets containing at least 20 g/day soy protein + 80 mg/day isoflavones *vs.* PCB diets	BP and Lipid profile	↓ BP, TC and non-HDLc and ↑ HDLc.
Flavonoids (Isoflavones)	Clerici *et al.* (2007) [74]	4	Ctrl, PA (8 weeks)	(62) Hypercholesterolemia	80 g serving/d (33 mg/day isoflavones + negligible soy protein + led to a serum isoflavone concentration of 222 +/- 21 nmol/L) *vs.* Ctrl group	Lipids profile, hsCRP, urinary 8-iso-PGF2α, and EF	↓ LDLc, TC
Flavonoids (Isoflavones)	Meyer *et al.* (2004) [75]	3	PCB, X (5 weeks, without washout)	(23) Mildly hypercholesterolemic and/or hypertensive	Soy-based milk (30 g/day soy protein + 80 mg/day isoflavones) + yoghurt (treatment) *vs.* equivalent dairy products (Ctrl)	BP, arterial compliance, lipid profile, fatty acids	No significant effect
Flavonoids (Isoflavones)	Jenkins *et al.* (2002) [76]	3	1B (1 month, 2 weeks washout)	(41) Postmenopausal women with hypercholesterolemia	A low-fat dairy food Ctrl diet, high- (50 g soy protein and 73 mg isoflavones/day), low- (52 g soy protein and 10 mg isoflavones/day) isoflavone soy food diets	BP, lipids profile, oxidized LDL, calculated CAD risk	Soy diets ↓ TC estimated CAD risk, TC/HDLc, LDLc/HDLc, ApoB/A-I. Blood lipid and BP changes, the calculated CAD risk ↓ with the soy diets
Flavonoids (Isoflavones)	Blum *et al.* (2003) [77]	4	2B, PCB, X (6 weeks, 1 month washout)	(24) Postmenopausal women with hypercholesterolemia	25 g/day soy protein *vs.* PCB	Vascular inflammation biomarkers	No significant effect

Table 3. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Isoflavones)	Teede *et al.* (2006) [78]	3	Ctrl, X (3 months)	(41) Hypertensive postmenopausal	Soy cereal (40 g/day soy protein + 118 mg/day isoflavones) *vs.* gluten PCB cereal	BP, arterial function	↑ 24 hour HR, area under curve of 24 h SBP
Flavonoids (Isoflavones)	Cicero *et al.* (2013) [79]	4	Ctrl, 1B prospective study with PA (12 weeks)	(40) Mildly dyslipidemic postmenopausal women	60 mg/day soy isoflavones + 500 mg/day berberine *vs.* PCB (1 tablet/d)	BP, HOMA, lipids profile, metalloproteinase	Isoflavones-berberine experienced a significant improvement in plasma lipid and metalloproteinase serum levels.
Flavonoids (Isoflavones)	Curtis *et al.* (2013) [80]	5	2B, PCB, PA (1 year)	(180) Postmenopausal women with T2D	27 g/day flavonoid-enriched chocolate (containing 850 mg flavan-3-ols [90 mg epicatechin] + 100 mg isoflavones [aglycone equivalents)] /d) *vs.* PCB.	Intima-media thickness of the common carotid artery, pulse wave velocity, augmentation index, BP, and vascular biomarkers	Only pulse pressure variability improved
Flavonoids (Isoflavones)	Curtis *et al.* (2013) [80]	5	PA, PCB (1 year)	(93) Postmenopausal women with T2D	27 g/day flavonoid-enriched chocolate (containing 850 mg flavan-3-ols [90 mg epicatechin] + 100 mg isoflavones [aglycone equivalents)] /d) *vs.* PCB.	HOMA and QUICKI, lipid profile, BP	Estimated 10-year total coronary heart disease risk (derived from UK Prospective Diabetes Study algorithm) was attenuated after flavonoid intervention
Flavonoids (Isoflavones)	Chan *et al.* (2008) [81]	5	2B, PCB (12 weeks)	(102) Prior ischemic stroke	80 mg/day isoflavone supplement *vs.* PCB	EF, nitro-glycerine-mediated dilatation, BP, HR, CHO metabolism, haemoglobin A1c, and oxidative stress biomarkers	↓ serum hsCRP and improved brachial EF in patients with clinically manifest atherosclerosis

Table 3. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Isoflavones)	Webb *et al.* (2008) [82]	4	2B, PA (5 days)	(71) Subjects with CAD	Isoflavone-intact soy protein (75 mg/day of isoflavones) *vs.* isoflavone-free PCB	Stimulated coronary blood flow, Basal and stimulated coronary artery luminal diameters	No significant effect
Flavonoids (Isoflavones)	Fanti *et al.* *(*2006) [83]	3	2B,Crtl, prospective, pilot study (8 weeks)	(32) ESRD patients with systemic inflammation	Nutritional supplements (soy groups) containing 26–54 mg isoflavones aglycones *vs.* isoflavone-free milk-based supplements (Ctrl group)	Inflammatory biomarkers	Inverse correlation between blood isoflavones levels and CRP, positive correlation between blood isoflavones levels and IGF-1
Flavonoids (Procyanidins)	Ras *et al.* (2013) [84]	5	2B, PCB, PA (8 weeks)	(70) Healthy subjects	300 mg/day Grape Seed Extract *vs.* PCB	BP	No significant effect
Flavonoids (Procyanidins)	Yubero *et al.* (2013) [85]	3	2B, PCB, X (56 days)	(60) Healthy subjects	700 mg/day the Grape Extract (Eminol®) *vs.* PCB	CVD risk and oxidative stress markers	↓TC, LDLc and ↑ TAC and vitamin E.
Flavonoids (Procyanidins)	Asher *et al.* (2012) [86]	5	2B, PCB, four-period X (3.5 days, 4 days washout)	(21) Pre-hypertensive or mildly hypertensive adults	Hawthorn Extract (1000, 1500, and 2500 mg/day) *vs.* PCB	EF and nitric oxide release	No significant effect

Table 3. *Cont.*

Group (Class)	Author/Date	Jadad Score	Design (Follow up)	(n) Population	Intervention	Outcomes	Significant Results
Flavonoids (Procyanidins)	Liu *et al.* (2004) [87]	3	PCB, 2B, PA (12 weeks)	(58) HT subjects	100 mg/day Pycnogenol *vs.* PCB	Endothelin	↓ Calcium antagonist nifedipine. ↓ endothelin-1 concentration and ↑ of 6-keto prostaglandin F1a.
Flavonoids (Procyanidins)	Enseleit *et al.* (2012) [88]	5	2B, PCB, X (8 weeks, 2 weeks washout)	(23) Patients with stable CAD	200 mg/day Pycnogenol *vs.* PCB	EF, oxidation and inflammatory markers, platelet adhesion and 24 h BP	EF improvement. ↓ 8-iso-PGF2α
Flavonoids (Procyanidins)	Mellen *et al.* (2010) [89]	3	2B, PCB, X (4 weeks, 4 weeks washout)	(50) Patients with CAD	1300 mg/day muscadine grape seed *vs.* PCB	EF, oxidation and inflammatory markers, antioxidant status	No significant effect
Flavonoids (Procyanidins)	Tauchert *et al.* (2002) [90]	3	2B, PCB (16 weeks)	(209) Chronic stable heart failure patients	1800 mg/day crataegus extract WS 1442 or 900 mg/day crataegus extract WS 1442 *vs.* PCB	Typical heart failure symptoms	Typical heart failure symptoms as rated by the patients were ↓ to a greater extent

[a]Soy extract contains: contains 17.5 mg soy isoflavones consisting of 5.25 mg glycitin, 8.75 mg daidzein, and 3.5 mg genistein; [b] 44 mg of daidzein, 16 mg of glycitein, and 10 mg of genistein; [c] Soy groups in three formats: Protein powder (54mg isoflavones), Cereal-like product (26mg isoflavones), energy bar (26 mg isoflavones). 8-iso-PGF2α, 8-iso-prostaglandin F2α; 1B, one-blind, 2B, double-blinded; ACN, anthocyanins; Apo, apolipoprotein; BMI, body mass index; BP, blood pressure; CAD, chronic artery disease; CAT, catalase; CHF, chronic heart failure; CHO, carbohydrate; CRP, C-reactive protein; hsCRP, high sensitivity c-reactive protein; Ctrl, control, CVD, cardiovascular disease; DXA, Dual-energy X-ray absorptiometry; EF, endothelial function; EGCG, epigallocatechin gallate; ESRD, European and North American end-stage renal disease; FM, fat mass; FFM, fat-free mass; HDLc, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment; HR, heart rate; HT, hypertension; sICAM, soluble intercellular adhesion molecule; IGF-1, insulin-like growth factor-1; IR, insulin resistance; IL, interleukin; LDLc, low-density lipoprotein cholesterol; MDA, malonaldehyde; MPFF, micronized purified flavonoid fraction; MPI, milk protein isolate; NTG, nitro-glycerine-mediated dilation; PA, parallel design, PAI-1, plasminogen activator inhibitor-1; PCB, placebo, PBMCs, peripheral blood mononuclear cells; PS, plant sterols; PWV, pulse wave velocity; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; SOD, superoxide dismutase; TC, total cholesterol; TGF, transforming growth factor; T2D, type 2 diabetes; VCAM, soluble vascular cellular adhesion molecule; vWf, von Willebrand factor; X, crossover design. *Included after proofreading.

3.1. Simple Phenols

3.1.1. Stilbenes

Eight articles complied with requirements and their score varied from 3–5 and are included in Table 1. The age ranged from 20 until 83 years, with sample sizes of 19–166 participants. Two studies included an acute intervention design and were carried out in overweight/obese men and post-menopausal women with elevated BP [19,20]. Participants consumed three doses of resveratrol (RSV) (30 mg, 90 mg or 270 mg) or 75 mg of trans-resveratrol [20] and one hour after supplementation, they determined possible improvement of EF. Wong *et al.* [19] observed that EF increased more with the highest dose and later confirmed the increase of flow mediated dilation (FMD) with a 75 mg dose in an acute and a long term study [20]. One study was developed in healthy smoker subjects [21], testing the efficacy of RSV (500 mg/day) on anthropometric parameters and CHO metabolism, apart from lipid profile, markers of inflammation and oxidative stress during 60 days. Thus, after RSV supplementation, hsCRP and decreased, while antioxidant status (TAS) increased. Effects of short-term oral supplementation (60 days) of RSV alone (20 mg/day) or with calcium fructoborate (CF) (20 RSV + 112 CF mg/day) in subjects with stable angina pectoris were recently evaluated by Militaru *et al.* [22], measuring lipids profile, hsCRP, left ventricular function markers and observed a stronger decreased in N-terminal prohormone of brain natriuretic peptide (NT-proBNP) after RSV+CF administration. Moreover, RSV alone presented the most significant decreases for TC and TAG, although, reduction of high sensitivity C-reactive protein (hsCRP) was greater using CF treatment (112 mg/day). Tomé-Carneiro and colleagues [23–26] reported four studies with grape extract containing RSV combination (GE-RES), comparing with grape extract alone (GE). In 2012, they developed a clinical trial in primary prevention of CVD patients. After six months with 350 mg/day GE or 350 mg/day GE-RES possible changes in lipid profile and oxidized LDL(oxLDL) were assessed [24] and then, doses were doubled for the next six months. LDLc, apolipoprotein B (ApoB), oxLDL, and oxLDL/ApoB ratio decreased in the GE-RES group, whereas non-HDLc (total atherogenic cholesterol load)/ApoB ratio increased. Moreover, they evaluated the effect in inflammatory and fibrinolytic biomarkers after one year [26]; many improvements were observed: hsCRP, tumour necrosis factor alpha (TNF-α), plasminogen activator inhibitor 1 (PAI-1), interleukin (IL) IL-6/IL-10 ratio, and soluble intercellular adhesion molecule (sICAM) significantly decreased, and IL-10 and adiponectin increased. . In 2013, they evaluated the same doses (350 mg/day GE or 350 mg/day GE-RES), for the first six months and double for the following six months in patients with T2D, HT and stable coronary artery disease (CAD) [25], as well as in patients with stable CAD alone [23]. Peripheral blood mononuclear cells (PBMCs),inflammatory andfibrinolytic biomarkers were assessed in both studies; the pro-inflammatory cytokines CCL3, IL-1β and TNF-α expression levels, were significantly reduced and transcriptional repressor LRRFIP-1 expression increased in PBMCs from T2D, HT and stable CAD patients taking the GE-RES extract [25]. In the other trial, the GE-RES group showed an increase of the anti-inflammatory serum adiponectin and PAI-1 decreased. In addition, six key inflammation-related transcription factors were predicted to be significantly activated or inhibited, with 27 extracellular-space acting genes involved in inflammation, cell migration and T-cell interaction signals presenting down regulation in PBMCs from stable CAD patients [23].

RSV has been shown to exert its protective effect against cardiovascular disease but it is necessary to reiterate that these data derive from cell culture or small animal model systems, with no reports on long-term health or survival in humans or alternate animal models [91]. It is well known that impaired FMD is recognized as an independent risk factor for the development of CVD [92,93]. Several authors investigated the acute resveratrol supplementation effect in overweight/obese individuals with mildly elevated BP, thus these subjects present cardiovascular risk [94,95]. Wong *et al.* [19] observed improvements in FMD were correlated with a dose-related increase in plasma RSV concentrations and following up their research, they confirmed the effect of RSV on FMD [20]. However, more long-term interventions are required. The other studies assessed long-term administration, until one year. Generally, trials obtained a decrease in total cholesterol (TC), triglycerides (TAG), C-reactive protein (CRP), CCL3, IL-1β, sICAM, NT-proBNP, TNF-α expression, LDLc, ApoB, LDLox and LDLox/ApoB ratio, as well as adiponectin, non-HDLc/ApoB ratio, IL-10 in different type of subjects [21–26].

Reports by Tomé-Carneiro *et al.* [23–26] focused on PBMCs, inflammatory andfibrinolytic biomarkers, lipid profile, and oxLDL concentration. Prior studies have shown increased mitochondrial production of ROS in PBMCs, endothelial cells, and other cell types in diabetes, suggesting systemic mitochondrial dysfunction [96,97]. Hartman *et al.* [98] observed higher basal, maximal, and uncoupled oxygen consumption in the diabetic patients, findings that are consistent with prior work showing increased mitochondrial ROS production in PBMCs. This suggests how serious complications may be in T2D subjects. Results by Tomé-Carneiro *et al.* [23,24] on transcriptional levels appear interesting; nevertheless, we have not found further similar interventions confirming these results.

3.1.2. Catechols

Two articles about catechols were selected (Table 1). According to the Jadad scale, such studies obtained values from 4–5. In 2008, Alwi *et al.* [27] assessed effects of curcumin on lipids profile in 75 acute coronary syndrome (ACS) patients (45–73 years). The efficacy was measured using different doses (45 mg/day, 90 mg/day or 180 mg/day) during two months, reporting higher effects in TC, LDL reduction and an increase in HDL with the lower dose, but changes were not significant in respect to placebo. Recently, a study [28] also evaluated the efficacy and safety of curcumin extract (750 mg/day) as an intervention agent for reducing the risks for atherogenesis in 240 T2D patients with a mean age of 61 years, by means of parameters such as BP, anthropometry, lipids profile, adiponectin, leptin, CHO metabolism, uric acid, and pulse wave velocity (PWV). After six months, curcumin treatment significantly reduced PWV, homeostasis model assessment (HOMA), TAG, acid uric, leptin and abdominal obesity, as well as significantly elevated values of adiponectin.

Alwi *et al.* [27] developed the first study to evaluate the effect of curcumin on the lipid profile of patients with ACS), thus the antecedents are described in *in vitro* and *in vivo* animal models. Results did not change significantly when comparing with placebo group. A meta-analysis based on five clinical trials in relation to curcumin on blood lipids concentration, indicated a non-significant effect of curcumin on the lipid profile when considering heterogeneous populations including healthy subjects, obese dyslipidemic patients, elderly subjects with established acute diagnosis of Alzheimer's disease, ACS and patients with T2D [99]. Chuengsamarn *et al.* [28] also assessed lipid profiles and did not find significant statistic differences compared with placebo. Ramirez-Boscá *et al.* [100] observed

that a daily treatment with curcumin extract could decrease significantly the LDLc and ApoB concentrations and increase the HDL and ApoAI in healthy subjects. However, due to lack of sufficient data we cannot recommend curcuminoids for improvement lipids profile in healthy and unhealthy subjects until further solid evidence is obtained.

On the other hand, after six months of curcumin intervention [28], PWV, HOMA, TAG, uric acid, abdominal obesity and leptin decreased, in addition to adiponectin increase. In addition, curcumin was well tolerated, with very few adverse effects. Agreeing with that, curcumin administration has been demonstrated, in *in vitro* and *in vivo* animal models, to elevate adiponectin and to decrease leptin levels [101,102], and oxidative stress in rabbits [14].

Due to its benefits and safety, Chuengsamarn *et al.* [28] proposed that curcumin extract might be used as anti-atherosclerotic in T2D populations. We propose however to replicate these results in other populations, since this study was performed in a Thai population and high variabilities of physical activity and diet among populations may exist that affect study results. Moreover, there are not enough studies in humans for recommending curcumin against T2D.

3.1.3. Beer or Wine Polyphenols

Four of the studies were focused on the effects of polyphenols derived from beer or wine; all were in subjects with risk of CVD (55–75 years) (Table 1). They tested 280 mg of red wine polyphenols or 30 g/day of beer or wine (normal and dealcoholized) for four weeks [29–32]. Botden *et al.* [29] analysed BP and found no significant effect. Chiva-Blanch *et al.* [30–32] reported that after the beer and non-alcoholic beer interventions the number of circulating endothelial progenitor cells (EPC)-mobilizing factors increased, consumption of dealcoholized red wine decreased BP and alcohol increased IL-10 and decreased macrophage-derived chemokine concentration and that the phenolic compounds of red wine decreased the serum concentrations of ICAM-1, E-selectin and IL-6.

Botden *et al.* [29] studied the effects of polyphenols from wine on BP and found no effect except that, the use of dealcoholized red wine reduced BP. Moreover, phenolic compounds are related to decreases of inflammatory and vascular homeostasis biomarkers. Nevertheless, there is not strong evidence showing that consumption of beer or wine could help to improve risk of CVD; reports in the literature do not focus on a specific compound, regardless of alcohol content. A review by Rotondo *et al.* [103] states that wine in low quantities could be beneficial in regard to CVD, but notes possible bias in the publications reviewed. It is necessary to focus research on a specific compound in alcohol-containing products, when assessing for potential benefits in CVD.

3.2. Polyphenols

The different research equations resulted in 59 articles related to different flavonoids and subclasses, such as anthocyanins (ACN), flavonols, flavanols, isoflavones and procyanidins. Eight of the studies were discarded because of lack of information in the abstract or inability to obtain the full-text version. The results are presented in groups according to their class in Tables 2 and 3.

3.2.1. Anthocyanins

Five publications were related to ACN (Table 2). Quality scores for these studies ranged from 4 to 5 in the Jadad scale. Two of the studies were exclusively in women between 23 and 58 years [33,34] (sample sizes from 31 to 57) and one, in 31 hypertensive men aged between 35 and 51 years [35]. The study developed by Dohadwala and colleagues [36] was in patients with CAD using an acute and a chronic approach.

The doses of ACN provided were from 500 to 640 mg/day [97,98] alone or as juice or blended drink including 94 mg/day [36], 983.7 mg/day and 840.9 mg/day [33], respectively, during periods from 14 days to 4 weeks.

The main outcomes in these studies were related to BP, lipid profile, CHO metabolism, inflammatory and oxidative stress biomarkers, platelet reactivity and vascular function.

One of the studies reported an increase of HDLc and blood glucose after the ACN intake, but no effects on oxidative stress biomarkers [35]. On the other hand, Kuntz *et al.* [33] reported an increase on superoxide dismutase (SOD) and catalase (CAT) and a decrease of malonaldehyde (MDA) after the ACN ingestion. The other studies did not find any significant effect when analysing BP, CHO metabolism, lipid profile, inflammatory biomarkers, platelet reactivity [34] or vascular function [36].

A recent systematic review has shown the effectiveness of anthocyanins in decreasing CVD risk [104]. Nevertheless, in this review we found that improvement of risk factors related to CVD such as BP, lipids profile, CHO metabolism, inflammatory, oxidative stress biomarkers, and platelet reactivity were not consistent. Hassellund *et al.* [35] reported modifications in lipid and CHO metabolism, but this result was not supported in the other investigations, as was the case with oxidative stress as well. There is not strong evidence supporting that anthocyanins help to decrease risk of CVD and further studies are required, thus, the grade of recommendation according to the SIGN guidelines is B.

3.2.2. Catechins

Four of the studies were related to catechins (Table 2), and the quality score assigned according to the Jadad scale was around 3–5. Two of the studies were with healthy subjects between 32 and 69 years old [37,38] (sample size 52 and 64 in each one). In regard to the other two studies, one included 52 subjects with early atherosclerosis (mean of 42 years of age) [39], and the other included 240 subjects with visceral fat-type obesity aged around 25–55 years old [40].

In the studies with healthy subjects [37,38] and the one in visceral fat-type obesity [40], catechins were obtained from green and black tea, with doses between 583 mg and 3 g per day during periods between 4 and 14 weeks. The atherosclerotic subjects [39] were supplemented with 30 mL of epigallocatechin gallate (EGCG)-supplemented olive oil during 4 months.

The main outcomes in the healthy subjects [37,38] were related to CVD risk, such as inflammatory and endothelial biomarkers. Subjects with early atherosclerosis [39] were investigated in regards to endothelial function and inflammatory and oxidative stress status. The aims in the subjects with visceral fat-type obesity [40] were anthropometric measurements, body fat composition and CVD risk factors.

There were no significant effects of the use of catechins in healthy subjects when compared with placebo/control; however, there was a negative correlation between beta-carotene and the inflammation biomarkers, IL-6 and fibrinogen [38]. On the other hand, the intervention in visceral fat-type obesity showed significant decreases in body weight, body mass index (BMI), body fat ratio, body fat mass, waist circumference (WC), hip circumference, visceral fat area and subcutaneous fat area, systolic blood pressure (SBP) and LDL cholesterol [40]. Nevertheless, in patients with early atherosclerosis, there was no significant effect, but merging both, the control (olive oil) and the experimental group (olive oil and EGCG)) the endothelial function was improved [39].

Catechins were shown to be effective in reducing LDLc and TC, but there is no robust evidence in reducing CAD risk [105]; a dose of 583 mg of catechins in middle-aged subjects showed a significant effect reducing obesity related makers, such as body weight, BMI, body fat ratio, body fat mass, WC, hip circumference, visceral fat area, and subcutaneous fat area, SBP, LDLc. However, doses of 630 mg or 3 g did not benefit middle and older aged subjects. Furthermore, Widmer *et al.* [39] investigated the effects of olive oil with EGCG in endothelial function without significant effect; however, they found a significant improvement when they merged both study groups. Nevertheless, this result is attributable to olive oil compounds, independently of the EGCG content. Taking into account the different studies included in this review, we can conclude that there is no robust evidence to suggest a beneficial effect of tea catechins on prevention of CVD; consequently, the grade of recommendation according to the SIGN guidelines is B.

3.2.3. Flavanols

Fourteen investigations studied the effects of flavanols (Table 2); the Jadad quality scores were between 3 and 5. Only one study was in healthy males (mean 68 years, and with a sample size of 40) [41], while four papers were related to overweight adults (with one of them including two designs and thus treated as separated studies [45]), involving subjects from 40 to 64 years (n = 21–98) [42,43]. Furthermore, there were two studies in hypertensive subjects [46,47] with 52 and 19 patients respectively. The study developed by Flammer *et al.* [48] included 20 chronic heart failure (CHF) patients (58 years mean) with an acute and a long-term intervention. Heiss *et al.* [49] and Horn *et al.* [50] studied the effect of flavanol in 16 CAD patients (60 years mean) while Balzer *et al.* [51] designed a study in diabetic patients with an acute and long-term intervention (10–41 subjects between 50 and 80 years).

Four of the studies [42,45,48,51] used a short-term approach looking for the acute response of flavanols; they tested cocoa or chocolate in amounts from 624 mg to 963 mg/day. Further, the other studies tested the flavanols contained in chocolate in longer interventions, from 4 to 6 weeks in doses from 33 to 1052 mg/day [43,44,46].

The aim of the study in healthy males [41] was to determine the effect on the endothelial function and in the soluble cellular adhesion molecules, without significant effects. In regards the studies with overweight [42,43,45], hypertensive [46,47], CHF [48], CAD [49,50] and diabetic patients [51], the main outcomes were related to EF and BP. Furthermore, Grassi *et al.* [47] studied the effects on lipids profile and IR.

The results in overweight, HT, CHF, CAD and diabetic patients showed a consistent improvement in EF when comparing different doses of flavanols *vs.* placebo/control [42–45,47–51].

When testing flavanols in BP, Faridi *et al.* [29] found a significant decrease in BP in overweight adults in an acute intervention with two different products using >800 mg/day of flavanols. Additionally, BP decreased significantly in hypertensive subjects using flavanol-rich chocolate during 15 days [47] and in CAD patients [49] with 375 mg twice daily during 30 days. However, this result was not consistent when using doses between 33 and 1052 mg/day of flavanones during six weeks [46] or 814 mg/day during four weeks [44]. Besides, Berry *et al.* [42] found out that cocoa flavanols could attenuate the increase of BP after exercise. The effects on IR were investigated in two studies, finding a significant improvement [43,47].

Flavanol-rich chocolate and cocoa products have shown a small but statistically significant effect in lowering blood pressure by 2–3 mm Hg in the short term [101], in addition Khawaja *et al.* [106] suggest that there is ample evidence in support of the beneficial effects of cocoa/dark chocolate on CHD risk. Summary of the evidence showed benefits of cocoa flavanols in BP and EF, in overweight adults [42–46], hypertensive subjects [47], in CHF [48], in CAD [49,50] and in T2D patients [51] utilizing different doses (149–963 mg/day). Additionally, Balzer *et al.* [51] reported a dose-response in an acute intervention. While the use of cocoa flavanols in BP and EF improvement have shown efficacy, further studies using flavanol-free controls could help to strength the evidence and long-term interventions may clarify the effect on CVD, thus the grade of recommendation according to the SIGN guidelines is B.

3.2.4. Flavonols

Five studies were focused on the effects of flavonols on CVD (Table 2); all of them obtained more than 3 points in the Jadad scale. Three of the publications were focused on healthy males [52–54] aged between 24 and 53 years (sample size between 12–27). The other two studies studied the effect on hypertensive subjects [52,55] (24–49 years; including 12 and 41 subjects, each one). Quercetin was the flavonol tested in two of the healthy subject studies at a dose of 1 g/day; Larson *et al.* [52] used it in an acute study and Conquer *et al.* [53] used it for 28 days; both of the authors looked for effects in BP and vascular markers without effect. Moreover, Suomela *et al.* [54] utilized oatmeal with 78 mg of flavonol aglycones from sea buckthorn for four weeks, and likewise did not find any significant effect.

The studies focused on hypertensive subjects looked for effects in BP, oxidative stress, angiotensin-converting enzyme and endothelin. Both, Edwards and Larson [52,55] found a significant reduction in BP in hypertension. Evidence around flavonol has been controversial; previous meta-analyses has associated its consumption with lower rates of CHD [8] or a reduction in risk of stroke [107,108], but reports from other authors do not support the protective role against CHD [10]. In the present review, we have found that doses of 1 g/day of quercetin or an oatmeal with 78 mg of aglycones of quercetin did not shown effects on diverse CVD risk markers, such as endothelin, BP or oxidative stress. Nevertheless, an acute intervention with 1095 mg/of quercetin and a long-term intervention (28 days) with 730 mg/day seems to be effective at reducing BP in hypertensive men [52,55], but not on other oxidative stress or endothelial function markers. We can conclude that there is no effect of flavonols in CHD, thus the grade of recommendation according to the SIGN guidelines is B, but since it seems that flavonols are effective at reducing BP in hypertensive men, further analysis in greater cohorts are needed.

3.2.5. Isoflavones

Thirty papers were related to the consumption of isoflavones (Table 3), the Jadad scores were above three points. Five of the studies were in healthy subjects between 20 and 53 years (sample size 22–205) [56,57,59,60,68]. Most of the studies were developed in postmenopausal women (45–92 years) with normal weight [61–67], overweight and obese [69–71], with BP alterations [78,109], dyslipidaemias [76,77,79] or T2D [80], including from 40 to 350 participants. Other authors also took men in account [58,72–75]. One study investigated in 102 subjects prior to ischemic stroke [81] (mean 66 years), another in 71 subjects with CAD (mean 58) [82] and Fanti and colleagues studied the effect on patients with systemic inflammation [83].

The doses of isoflavones employed in healthy subjects and postmenopausal women were between 40 and 118 mg/day, with lengths between 17 days to two years. The intervention in postmenopausal women with T2D used 100 mg/day of isoflavones for a one-year period. The subjects with ischemic stroke consumed 80 mg/day of isoflavones for 12 weeks. Patients with CAD included 75 mg/day during five days. Subjects with systemic inflammation included doses between 26–54 mg/day of isoflavones aglycones [56–80,109].

The main outcomes established in the articles of healthy, overweight and obese subjects were related to anthropometry [66], body composition [63,70,71,109], lipid profile [56–60,62,63,65–70], BP, CHO metabolism [60,62,63,66,70], and inflammatory [57,59,63,64,67], oxidative stress [62] and vascular homeostasis biomarkers [59,62,64] and only one in atherosclerosis progression [61]. Besides, the studies in hypertensive and dyslipidemic patients aimed on lipids profile [73,74,76,79], BP [73,75,76,78,79], oxidative stress [72,74,76], endothelial function [75,78] and just one in vascular inflammation biomarkers [77]. Two articles from Curtis *et al.* [80,110] in postmenopausal women with T2D looked for effects in HOMA, QUICKI, lipids profile, intima-media thickness of the common carotid artery, pulse wave velocity, augmentation index, BP, and vascular biomarkers.

The results reported in healthy, overweight and obese patients related to anthropometry and body composition were without significant effects of isoflavones. Moreover, in relation with lipids profile, a study reported lower ratios of TC/HDLc, LDLc/HDLc, and ApoB/Apo A-I [56] and another a decrease in TC and LDLc [71]. Besides, Sanders *et al.* [57] found significant improvements in HDLc and Apo-A; however, seven studies did not find any significant effect in plasma/serum lipids [58–60,63,65–67,69]. Fasting glucose, insulin, and HOMA were reduced in three studies [59,60,66] but results from other authors were not consistent [63,69,70]. The only author that studied BP [63] did not find any significant change. Atteritano *et al.* [62] reported significant improvements in isoprostanes (8-iso-PGF2α), sICAM-1 and soluble vascular cell adhesion molecule-1, while Atkinson *et al.* [59] and Sanders [57] investigated PAI-1 and other authors [72,74] 8-iso-PGF2α without significant results. One study showed a decrease in thromboxane A2 [63] and another in CRP [64]. The study of Liu *et al.* [67] aimed in inflammatory markers showed no effect. Hodis *et al.* [61] analysed atherosclerosis progression finding no positive effects. Furthermore, in subjects with BP alterations and dyslipidaemias, there were no significant changes in CHO metabolism [79,80], oxidative stress or inflammatory biomarkers [72,74,76,77]. In relation to BP, Sagara *et al.* [73] and Teede *et al.* [78] reported improvements; nevertheless, these results were not consistent with the results obtained by other authors [75,76,79,80]. Moreover, when lipid profile were analysed, a decrease in total cholesterol,

LDLc and a decrease was observed [73,76,79] but Meyer *et al.* [75] and Thorp *et al.* [58] reported no significant change. Many authors [52,56,57,108] described no significant effects on endothelial function; nonetheless, Jenkins *et al.* [76] reported a lower calculated risk of CAD. The intervention in subjects' prior ischemic stroke [81] found a reduction in hsCRP and improved the EF, beside CAD patients [82] showed no effect on EF. Fanti *et al.* [83] found a significant inverse correlation between isoflavones and CRP.

The use of isoflavones on the prevention of CVD has been associated with the capacity of these compounds to attenuate alterations in lipid profile and inflammatory markers [111,112]. Furthermore, the American Diet Association recommended consumption of soy protein containing isoflavones in high-risk populations with increased total cholesterol and LDLc. Additionally, data from two cohorts [113,114] showed that isoflavones consumption is associated with a lower risk of cardiovascular disease in women. Interestingly, we found that doses above 50 mg/day in healthy subjects improved lipids profiles [56,57,68], but a lower dose for 12 months did not show effect on CVD risk factors [45]. Additionally, normal weight postmenopausal women did not improve the lipid profile in many of the studies in which they included as outcome [60,63,65–67,69]. Nevertheless, the response of dyslipidemic postmenopausal women was positive when treated with doses between 60 and 80 mg/day of isoflavones, decreasing TC, HDLc and ratios TC/HDLc, LDLc/HDLc and ApoB/A-I [76,79].

Glucose, insulin and HOMA measurement are important due to its relationship in development of CVD. Although, these indicators were reduced in normal weight subjects [41,43,49], isoflavones in high doses (60–100 mg/day) showed no benefit on overweight/obese postmenopausal women [63,69,70,79].

BP was only measured by Garrido *et al.* [63] in healthy subjects without significant changes. A different panorama was shown in subjects with BP and dyslipidaemias, while doses above 80 mg improved BP [73,78], doses below did not have any effect [75,76,79,80].

The research focused on inflammatory and oxidative stress biomarkers in healthy subjects gave inconclusive results, markers such as fibrinogen and PAI-1 were not reduced, but only two authors measured them [57,59]. Thromboxane A2, CRP, and 8-iso-PGF2α were markers that also responded effectively to isoflavones intervention, but were not taken in account in all the investigations [57,59,60,62–64,67,71]. Moreover, Liu *et al.* [109] focused his research in measuring inflammatory markers after an intervention with soy or milk protein plus 100 mg of isoflavones without significant effects. This lack of consistent results was already showed by Dong and colleagues [114] in a meta-analysis including 14 trials that analysed soy foods with isoflavones concluded that there is insufficient evidence that soy isoflavones significantly reduce CRP concentrations in postmenopausal women.

Dysfunction of the vascular endothelium has shown to be an early step prior to development of atherosclerosis [115], its prevention is vital for the maintenance of vascular health. Some authors reported no EF improvement [65,74,75,80], indeed, Webb *et al.* [82] conclude a lack of effect on EF in their intervention; however, the inclusion of more women to perform a specific gender analysis could give different results. Nevertheless, Chang *et al.* demonstrated that 12-week isoflavone treatment improved brachial FMD in patients with clinically manifest atherosclerosis, thus reversing their endothelial dysfunction status. In this regard, Li *et al.* [9] developed a meta-analysis measuring isoflavones on vascular endothelial function in postmenopausal women and conclude that oral isoflavone supplementation does not improve endothelial function in postmenopausal women with

high baseline FMD levels but leads to significant improvement in women with low baseline FMD levels. Furthermore, Pase *et al.* [116] determined that soy isoflavone supplementation provides an effective means of reducing arterial stiffness, but this review showed to be biased.

Lastly, the use of isoflavones in body composition and anthropometric measurements showed no effect in most of the interventions that included study of outcomes [63,66,67,69,70]. In summary, the need of further studies with greater population sizes are necessary; the effect of the inflammatory process and endothelial function in postmenopausal women on are possibly the most interesting areas of study. The grade of recommendation according to the SIGN guidelines is B.

3.2.6. Procyanidins

Eight studies from the search were related to procyanidins (Table 3), with a Jadad score above three points. Two interventions were in healthy subjects between 34 and 75 years [84,85], sample size 60 and 70, in each one. Asher *et al.* [86] and Liu [87] studied the effects of procyanidins in HT and the three other studies were in CAD (18–73 years, sample size of 23 and 50) and the last study included 209 chronic stable New York Heart Association class-III heart failure subjects [90].

The interventions in healthy subjects used 300–700 mg/day of grape extracts with a length of eight weeks in both [84,85]. In HT subjects, the hawthorn extract was investigated in a short tem study of three and a half days in doses of 1 g, 1.5 and 2.5 g [86]. In the other study they used 100 mg/day of Pycnogenol® during 12 weeks [87]; this product was also utilized at a dose of 200 mg/day in stable CAD patients for eight weeks [88]. Besides, muscadine grape seed was used for four weeks in doses of 1300 mg/day [89]. The subjects with stable CHF were treated with 1800 mg of crataegus extract WS 1442 or 900 mg of crataegus extract WS 1442 or with placebo for 16 weeks [90].

In healthy subjects, the outcomes were related to BP, CVD and oxidative stress biomarkers, in hypertensive subjects to endothelial function, in CAD to endothelial function, inflammatory and oxidative stress biomarkers. In patients with stable CHF, the aim was related to typical heart failure symptoms [84–88,90].

There were no significant effects in BP in healthy subjects [84]. However, Yubero *et al.* [85] reported a significant decrease in TC, LDL and an increase in TAC and vitamin E. Furthermore, in hypertensive subjects, Asher *et al.* [86] did not find significant effects, while on the contrary Liu *et al* [87] found a reduction of endothelin-1 concentration and an increase in 6-keto prostaglandin F1a. Moreover, the use of Pycnogenol in CAD also showed an improvement of endothelial function and a reduction of 8-iso-PGF2α [88]. The muscadine grape seed did not show any significant effect [89] and the crataegus extract seemed to reduce the typical heart failure symptoms [90].

Procyanidins are compounds that can stabilize membranes, preventing their disruption by chemical and biological agents, thus mitigating oxidative stress and the activation of proinflammatory signals, factors related to development of CVD. Evidence found in the literature reviewed is not consistent. In healthy subjects, there was no effect on BP [84] using 300 mg/day of grape seed extract. However, Yubero *et al.* [85] reported improvement on CVD risk and oxidative stress markers with 700 mg/day. Besides, in hypertensive subjects, one study including different doses showed no improvements in EF or NO release, while another with 100 mg of Pycnogenol® reduced the concentration of endothelin and

6-keto prostaglandin F1a. The same compound was utilized in a dose of 200 mg/day in patients with stable CAD with improvements in EF and a decrease in isoprostanes. However, Mellen *et al.* [89] studied the effect of muscadine grape seed (1300 mg) without further effects. Finally, Tauchert *et al.* [90] included two different extracts from crataegus in typical heart failure symptoms with a positive decrease as rated by patients. In this regard, there is insufficient evidence to determine if extracts containing procyanidins could improve CVD risk; further investigations are necessary for more homogenous outcomes and greater populations; therefore, the grade of recommendation is C.

4. Limitations and Future Perspectives

Certain limitations need to be considered. Firstly, MeSH terms are not often used by researchers. Such specific terms must be taken into account when articles are drafted and assuring a good indexation and more visibility, facilitating the evidence valuation. Secondly, the application of resources such as CONSORT (Consolidated Standards of Reporting Trials) statement or Jadad scale is highly scarce. Moreover, the existence of checklists helps authors and editors to improve the reporting of RCTs and consequently provides scientific quality in data reports. We consider that a clinical trial is reliable when it is at least randomized and blinded. In addition, the trials included in this review have high levels of heterogeneity, making it more difficult to draw concrete conclusions in relation to types of subjects, form of analysed product or its combination with other compounds.

Future studies must show better designs to avoid the risk of bias usually associated with potential confounding variables such as other dietary or lifestyle factors. In addition, the dosages, polyphenol type, duration and frequency of consumption must be clear, giving the opportunity to assess the possible benefits to a specific compound. Long-term, double-blind, crossover, randomized clinical trials with specific clinical endpoints should be developed to guarantee the possible benefits of phenolic BAC.

In addition, the use of potent new technologies such as omics sciences *i.e.* transcriptomics, metabolomics, could help to elucidate the different mechanisms in which BAC are involved in CVD and its specific role.

5. Conclusions

The role of BAC as adjuvants in CVD is increasing and validation of its effects is essential. Evidence shows that some polyphenols used as BAC such as flavonols are helpful in decreasing risk factors of CVD. However, it is necessary to develop better quality RCTs (crossover design, double-blinded, long term, placebo/controlled) as well as elaborate rigorous meta-analysis of existing evidence to support the effect of BAC on the prevention and treatment of CVD.

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Author Contributions

O.D.R., B.P.V., C.A.G., A.G. contributed to the planning of the search of the literature, designed the analysis and results presentation and created the tool for assessing the quality of the articles. O.D.R., B.P.V. were involved in the analyses of the articles. O.D.R., B.P.V. wrote the draft. All authors discussed and revised all drafts and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest. This paper will be part of the Oscar Daniel Rangel Huerta's doctorate that it is being carried out within the context of "Biochemistry and Molecular Biology" at the University of Granada.

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VALORACIÓN NUTRICIONAL

DATOS PERSONALES

Fecha de Ingreso: **Código:**

Nombre:

Edad:

ESTADO NUTRICIONAL

FECHA				
TENSIÓN ARTERIAL				
Frecuencia cardíaca (lat/min)				
Tensión sistólica (mmhg)				
Tensión diastólica (mmhg)				
ANTROPOMETRÍA				
Talla (m)				
Peso actual (kg)				
Peso habitual (kg)				
IMC (kg/cm^2)				
Perímetro cintura (cm)				
Perímetro abdominal (cm)				
Perímetro cadera (cm)				
Perímetro muslo (cm)				
PB (cm)				
PCT (mm)				
PCB (mm)				
PCA (mm)				
PCSe (mm)				
BIOIMPEDANCIA				
Agua corporal total (kg-%)				
Masa grasa (kg-%)				
Masa libre de grasa (kg)				
Masa muscular (kg)				
Masa ósea (kg)				
Edad metabólica (años)				
Grasa visceral (rating)				

HISTORIA MÉDICA

DATOS PERSONALES

Fecha de Ingreso: ☐☐☐☐☐☐ **Código:** ☐☐☐☐☐☐

Nombre: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Edad: ☐☐☐

Antecedentes personales: _____

_____

_____

Hiperuricemia ☐ **Dislipemias** ☐ **HTA** ☐ **DM** ☐

Sobrecarga articular ☐ **SAOS** ☐

Antecedentes Familiares: _____

_____

_____

Obesidad ☐ **Hiperuricemia** ☐

Dislipemias ☐ **HTA** ☐ **DM** ☐

Sobrecarga articular ☐ **SAOS** ☐

Quien: _____

Anamnsesis: _____

_____

_____

Exploración física: _____

_____

_____

Medicación actual: _____

_____

_____

Juicio clínico: _____

_____

_____

CUESTIONARIO ESTILOS DE VIDA

DATOS PERSONALES

Fecha de Ingreso: ☐☐☐☐☐☐ **Código:** ☐☐☐☐☐☐

Nombre: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Edad: ☐☐☐

PESO ACTUAL:		PESO HABITUAL:	
TALLA:		IMC:	
CONSTITUCIÓN:	Grande	Mediana	Pequeña
PESO			
MÁXIMO EDAD ADULTA:		MÍNIMO EDAD ADULTA:	

Hábitos de vida

Descripción y duración (días, horas y/o minutos)

Actividad principal: _____

_____

Actividad sedentaria (ver TV, leer, estudiar, estar sentado): _____

_____

Actividad moderada (caminar, ir en bici, nadar): _____

_____

Actividad intensa (correr, gimnasia, juego de pelota): _____

_____

Esfuerzo vigoroso y de mucha actividad (entrenamiento): _____

_____

Actividades en el hogar: _____

_____

HISTORIA DE SALUD

¿Cuándo comenzaron sus problemas de peso?

Infancia/adolescencia	☐	Edad adulta	☐
Con los embarazos	☐	Con la menospausia	☐
Al dejar de fumar	☐	Tras reposo/cirugía	☐
Tratamiento médico	☐		

	SI	NO
¿Ha realizado dietas anteriormente?	☐	☐
¿Los ha llevado a cabo bajo supervisión médica?	☐	☐
¿Cuántas dietas ha realizado en los últimos 2 años?		
Tiempo máximo que ha permanecido a régimen		
Kg perdidos		

Motivos que le conducen a perder peso

Salud física	☐	Estética	☐
Profesional	☐	Otros	☐

¿Por qué suele abandonar los regímenes?

Aburrimiento	☐	Por el trabajo	☐
Estancamiento	☐	Fuerza de voluntad	☐
Problemas de salud	☐	Falta de apoyo familiar	☐
No se lo toma en serio	☐	Le gusta comer	☐

¿Cómo reacciona su familia cuando realiza dieta?

Aprobación	☐	Comprensión	☐
Indiferencia	☐	Escepticismo	☐
Comentarios risueños	☐	Críticas	☐
Intolerancia	☐	Hostilidad	☐

¿Come alimentos entre comidas?

Casi nunca ☐ Algunas veces ☐ Frecuentemente ☐

¿Come alimentos fuera de casa?

Casi nunca ☐ Algunas veces ☐ Frecuentemente ☐

¿Cuando termina de comer la cantidad servida pide que le sirvan más?

Casi nunca ☐ Algunas veces ☐ Frecuentemente ☐

¿Fuma?

No fumo ☐ Algunas veces ☐ Fumo a diario ☐

¿Cuántos cigarrillos fuma al día?

Ninguno ☐ 1 a 5 ☐ 6 o más ☐

¿Bebe alcohol?

Nunca ☐ Rara vez ☐ 1 vez o más/semana ☐

¿Cuántas bebidas alcohólicas toma en cada ocasión?

Ninguna ☐ 1 a 2 ☐ 3 o más ☐

NECESITAMOS SABER DE DONDE SE HA SACADO ESTE CUESTIONARIO PARA ASEGURARNOS QUE PODREMOS PUBLICAR DESPUÉS

EMILIO VA A ENVIAR UN CUESTIONARIO UTILIZADO EN UN PROYECTO DE ENCUESTAS ANDALUZAS Y NOS ENVIARÁ UNOS CUESTIONARIOS QUE HAN SIDO RECOMENDADOS (OS ENVIARÉ LAS REFERENCIAS CUANDO LAS TENGA)

EMILIO VA A BUSCAR INFORMACIÓN PARA CALCULAR EL HEALTHY EATING INDEX (HEI)

IMPORTANTE PREFERENCIAS ALIMENTARIAS

CUESTIONARIO DE FRECUENCIA DE CONSUMO DE ALIMENTOS

Modificación de: JOSE M MARTIN-MORENO, PETER BOYLE, LYDIA GORGOJO, PATRICK MAISONNEUVE, JUAN C FERNANDEZ-RODRIGUEZ, SIMONETTA SALVINI and WALTER C WILLETT. Development and Validation of a Food Frequency Questionnaire in Spain. Int. J. Epidemiol..1993; 22: 512-519

NOTAS: Para rellenar este cuestionario deben hacer referencia al consumo de alimentos durante el último año. Los alimentos estacionales (algunas frutas y otros vegetales) que solo se consumen en esa estación deben de corregirse de manera que se refieran a consumo anual (por ejemplo, si se han consumido durante 3 meses, dividir su frecuencia de consumo en ese periodo por 4)

	Nunca o Raramente (< 1 mes)	1-3 por mes	1 por semana	2-4 por semana	5-6 por semana	1 por día	2-3 por día	4-5 por día	6+ al día
I. LÁCTEOS									
1. Leche entera (1 vaso o taza, 200 cc)									
2. Leche semidesnatada (1 vaso o taza, 200 cc)									
3. Leche desnatada (1 vaso, 200 cc)									
4. Leche condensada (1 cucharada)									
5. Yogur entero (Uno, 125 g)									
6. Yogur dietético, sin azúcar, desnatado, otros (Uno, 125 g)									
7. Requesón, cuajada, queso blanco o fresco (100 g)									
8. Queso cremoso o en porciones (Una porción)									
9. Queso semicurado o curado: manchego (1 trozo, 50 g)									
10. Natillas, flan, puding (Uno)									

	Nunca o Raramente (< 1 mes)	1-3 por mes	1 por semana	2-4 por semana	5-6 por semana	1 por día	2-3 por día	4-5 por día	6+ al día
11. Helados (1 cucurucho, vasito, bola)									
II. HUEVOS, CARNES, PESCADOS									
12. Huevos de Gallina (Uno)									
13. Pollo con piel (1 plato o pieza)									
14. Pollo sin piel (1 plato o pieza)									
15. Carne de cerdo como plato principal (1 plato o pieza)									
16. Carne de cordero como plato principal (1 plato o pieza)									
17. Carne de ternera como plato principal (1 plato o pieza)									
18. Carne de caza: conejo, codorniz, pato (1 plato)									
19. Hígado de ternera, cerdo o pollo (1 plato)									
20. Vísceras: callos, sesos, mollejas (1 ración, 100 g)									
21Embutidos: jamón, salchichón, salami, mortadela (1 ración, 50 g)									
22. Salchichas o similares (Una mediana)									
23. Patés, foie-gras (media ración, 50 g)									
24. Hamburguesas (Una, 100 g)									
25. Tocino, bacon, panceta (2 lonchas, 50 g)									
26. Pescado frito variado (1 plato o ración)									
27. Pescado blanco hervido o plancha: merluza, lenguado (1 ración)									
27bis. Pescado azul hervido o plancha: sardina, atún (1 ración)									
28. Pescados en salazón: bacalao, anchoas (media ración, 50 g)									
29. Pescados en conserva: atún, sardinas, arenques (una lata)									
30. Almejas, mejillones, ostras (1 ración, 100 g)									

	Nunca o Raramente (< 1 mes)	1-3 por mes	1 por semana	2-4 por semana	5-6 por semana	1 por día	2-3 por día	4-5 por día	6+ al día
31. Calamares, pulpo (1 ración, 100 g)									
32. Marisco: gambas, langosta y similares (1 ración, 100 g)									
III. VERDURAS Y LEGUMBRES									
33. Espinacas, acelgas cocinadas (1 plato)									
34. Col, coliflor, brócoli cocinados (1 palto)									
35. Lechuga, endivias, escarola (1 plato)									
36. Tomates (Uno mediano)									
37. Cebolla (Una mediana)									
38. Zanahoria, calabaza (Una o plato pequeño)									
39. Judías verdes cocinadas (1 plato)									
40. Berenjenas, calabacinos, pepinos (uno)									
41. Pimientos (Uno)									
42. Espárragos, alcachofas (1 ración o plato)									
43. Champiñones, setas (1 plato)									
44. Legumbres cocinadas: lentejas, garbanzos, judías pintas o blancas (1 plato mediano)									
45. Guisantes cocinados (1 plato)									
IV. FRUTAS									
46. Naranjas, pomelo, mandarinas (Una)									
47. Zumo de naranja natural (un vaso pequeño, 125 cc)									
48. Plátano (Uno)									
49. Manzana, pera (1 mediana)									
50. Fresas (1 plato o taza de postre)									

	Nunca o Raramente (< 1 mes)	1-3 por mes	1 por semana	2-4 por semana	5-6 por semana	1 por día	2-3 por día	4-5 por día	6+ al día
51. Cerezas (1 plato o taza de postre)									
52. Melocotón, albaricoque, nectarina, ciruela (Uno mediano)									
53. Kiwi (uno mediano)									
54. Mango, chirimoyo, caqui (1 mediano)									
55. Higos frescos (Uno)									
56. Sandía, melón (Una tajada o cala, mediana)									
57. Uvas (1 racimo mediano o plato de postre)									
58. Aceitunas (tapa o plato pequeño, aprox. 15 unidades pequeñas)									
59. Frutas en almíbar: melocotón, pera, piña (Dos mitades o rodajas)									
60. Frutos secos: Piñones, almendras, cacahuete, avellanas (1 plato o bolsita pequeña)									
V. PAN, CEREALES Y SIMILARES									
61. Pan blanco (Una pieza pequeña o 3 rodajas de molde, 60 g)									
62. Pan integral (Una pieza pequeña o 3 rodajas de molde, 60 g)									
63. Picos, roscos y similares (Una unidad, 3.5 g)									
64. patatas fritas (1 ración)									
65. patatas cocidas, asadas (Una patata mediana)									
66. Bolsa de patatas fritas (1 bolsa pequeña, 25-30 g)									
67. Arroz cocinado (1 plato mediano)									
67. Pastas: espagueti, macarrones y similares (1 plato)									
67bis. Pastas rellenas: Raviolis y similares (1 plato)									
VI. ACEITES Y GRASAS									
68. Aceite de oliva (1 cucharada)									

	Nunca o Raramente (< 1 mes)	1-3 por mes	1 por semana	2-4 por semana	5-6 por semana	1 por día	2-3 por día	4-5 por día	6+ al día
69. Otros aceites vegetales: girasol, maíz, soja (1 cucharada)									
70. Margarina añadida al pan o la comida (Una cucharada o untada)									
71. Mantequilla añadida al pan o la comida (Una cucharada o untada)									
72. Manteca (de cerdo) añadida al pan o la comida (Una cucharada o untada)									
VII. DULCES Y PASTELERÍA									
73. Galletas tipo María (1 galleta)									
74. Galletas con chocolate (1 galleta doble)									
75. Croissant, Donet (Uno)									
76. Magdalena, Bizcocho (Uno)									
77. Pasteles, tarta (unidad o trozo mediano)									
78. Churros (masa frita) 1 ración									
79. Chocolate, bombones (Una barrita o dos bombones, 30 g)									
80. Chocolate en polvo o similares (1 cucharada)									
VIII. BEBIDAS									
81. Vino blanco, tinto, rosado (1 vaso, 125 cc)									
82. Cerveza (Una caña o botellín 1/5, 125 cc)									
83. Brandy, ginebra, ron, whisky, vodka, aguardientes 40º (1 copa, 50 g)									
84. Refrescos con gas: cola, naranja, limón (ej. Cocacola, fanta, etc. (Uno, 250 cc)									
85. Zumo de frutas envasado(Una lata pequeña o vaso, 200 cc)									
86. Café (Una taza)									
87. Café descafeinado (Una taza)									
88. Infusiones (Una taza)									

	Nunca o Raramente (< 1 mes)	1-3 por mes	1 por semana	2-4 por semana	5-6 por semana	1 por día	2-3 por día	4-5 por día	6+ al día
89. Batidos lácteos (1 brick, 200 ml)									
IX. PRECOCINADOS, PREELABORADOS Y MISCELANEAS									
90. Croquetas (Una)									
91. Palitos o delicias de pescado fritos (Una unidad)									
92. Sopas y cremas de sobre (1 plato)									
93. Mayonesa (1 cucharada)									
94. Salsa de tomate (media taza)									
94. Picantes: tabasco, pimienta, guindilla (1/2 cucharadita)									
95. Sal (Una pizca o pellizco con dos dedos)									
96. Ajo (1 diente)									
97. Mermeladas, miel (1 cucharada)									
98. Azúcar (ej. En el café, postres, etc.) (1 cucharada)									

RECUERDO DE 24H

Nadia Slimani, Genevie`ve Deharveng, Ruth U. Charrondiere, Anne Linda van Kappel, Marga C. Ocke, Ailsa Welch, Areti Lagiou. Structure of the standardized computerized 24-h diet recall interview used as reference method in the 22 centers participating in the EPIC project. Computer Methods and Programs in Biomedicine 58 (1999) 251–266.

1. Información no dietética

Entrevistador	
Código:	
Fecha/hora:	
Entrevistado	
Código:	
Edad (años):	
Sexo (H/M):	
Peso (Kg):	
Talla (cm):	
Día recordado	
Especial (viaje, fiesta, celebración)	
Dieta (HTA, diabetes, vegetariano)	

2. Lista rápida

Número de comidas:			
Desayuno	**Hora:**	**Lugar:**	**Alimentos:**
Media mañana	**Hora:**	**Lugar:**	**Alimentos:**
Aperitivo	**Hora:**	**Lugar:**	**Alimentos:**

Almuerzo	**Hora:**	**Lugar:**	**Alimentos:**
Merienda	**Hora:**	**Lugar:**	**Alimentos:**
Aperitivo	**Hora:**	**Lugar:**	**Alimentos:**
Cena	**Hora:**	**Lugar:**	**Alimentos:**
Antes de acostarse	**Hora:**	**Lugar:**	**Alimentos:**

NOTAS: Seguir un **orden cronológico**, introducir primero los **alimentos genéricos** (Leche, Yogur, Lentejas, etc.) y **asociarlos a las comidas** del día (ocasiones de consumo de alimentos, OCA). Intentar **asociar acontecimientos** con comidas para facilitar el recuerdo (que hizo a medio día, que programa de TV vio en la noche, etc.). Elaborar **listas de chequeo** de alimentos fácilmente olvidables (Bebidas, pan, tapas, aperitivos, ensaladas, postres, azúcar, aderezos, etc.)

3. Descripción y Cuantificación

Comida	**Alimento**	**Descripción del Alimento**	**Receta**			**Método de cocinado**
			Ingredientes	**Nº comensales**	**Cantidades**	
Desayuno						
Media mañana						

Aperitivo						
Comida	**Alimento**	**Descripción del Alimento**	**Receta**			**Método de cocinado**
			Ingredientes	**Nº comensales**	**Cantidades**	
Almuerzo						
Merienda						
Aperitivo						
Comida	**Alimento**	**Descripción del Alimento**	**Receta**			**Método de cocinado**
			Ingredientes	**Nº comensales**	**Cantidades**	
Cena						
Antes de acostarse						

NOTAS:

1. Sobre la lista rápida ir concretando y describiendo los alimentos/recetas consumidos. Incluir marcas comerciales.
2. Recetas: desglosarlas en ingredientes y describirlos. Incluir nombres comerciales. Preguntar quién cocinó la receta y para cuantos comensales
3. Recetas: si es conocida describirla. Si no es conocida seleccionar una receta estándar lo más parecida posible modificándola con las sugerencias y aportaciones del entrevistado (tipo de

aceite, ingredientes que no utiliza, tipo de leche, etc.). Si no hay receta estándar requerir una descripción lo más detallada posible

4. **Cuantificación:** Utilizar el álbum de fotografías. Si no estuviera el alimento consumido utilizar por este orden: unidades de peso/volumen; medidas caseras; unidades estándar; porciones estándar; desconocido (a estudiar).Se debe de preguntar y anotar los siguientes datos cuando proceda: Número de unidades; Ración (porción) entera o fracción; Fotos (2ª ración); Rangos de incremento (25%); Medidas caseras. Foto o "en vivo"; Regla: Pequeño-Mediano-Grande. Mostrar la media. Pan: Modelos bidimensionales de rebanadas, barras, etc. Siempre que se pueda acudir al álbum de fotografías. Si no se puede cuantificar acudir a cantidades estándar. Es importante tener en cuenta la grasa absorbida por el alimento y si aprovecha las salsas con o sin pan.
5. **Para la cuantificación de recetas:** Cantidad de la receta como es consumida (acudir siempre que se pueda a las fotos); cuantificación de cada uno de los ingredientes en crudo y cocinados; parte comestible; densidad. Cálculo del peso de cada ingrediente como es consumido.

4. Lista de chequeo de alimentos que pueden olvidarse fácilmente

- Grasa y aceites
- Salsas
- Azúcares añadidos
- Aperitivos
- **Alimentos de no correcta referencia** (repasar)

5. Control de calidad

- ✓ **En diferentes etapas de la entrevista**
  - Valores finales insertados
  - Valores finales calculados
  - Ítems en blanco
- ✓ **Al final de la entrevista**
  - Suplementos
  - Medicación (vitaminas o minerales)
  - Adición de productos

Appendix table 1. Significant *time effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Time Main Effect		NPJ Final / NPJ Baseline		HPJ Final / HPJ Baseline		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
N-acetylputrescine	1.05	0.84	0.38	0.30	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	2.1255	0.7979	2.2234	0.6669
methyl glucopyranoside (alpha + beta)	0.51	1.56	2.13	6.52	0.0000	0.0000	0.0000	0.0036	0.0000	0.0000	0.8409	1.7935	0.4288	2.7941
N-acetylthreonine	1.08	1.14	1.21	1.28	0.0000	0.0000	0.0000	0.0015	0.0000	0.0000	0.8953	1.0871	0.9674	1.2362
1-methylimidazoleacetate	1.28	0.83	0.43	0.28	0.0000	0.0000	0.0000	0.0022	0.0000	0.0000	1.7708	0.7701	2.2588	0.6370
betonicine	0.31	1.66	2.07	11.01	0.0000	0.0000	0.0001	0.0059	0.0000	0.0000	0.6834	1.4148	0.2139	2.3553
N-methyl proline	0.99	1.49	2.66	4.01	0.0000	0.0000	0.0003	0.0131	0.0000	0.0000	0.5258	1.3968	0.5209	2.0881
scyllo-inositol	0.82	1.41	1.43	2.48	0.0000	0.0000	0.0028	0.0798	0.0000	0.0000	0.7414	1.0638	0.6050	1.4997
stachydrine	0.79	1.23	2.49	3.89	0.0000	0.0000	0.0002	0.0113	0.0000	0.0002	0.5200	1.2967	0.4118	1.6003
tryptophan betaine	1.15	1.21	0.53	0.55	0.0000	0.0000	0.0000	0.0036	0.0004	0.0091	1.4137	0.7464	1.6304	0.9021
ectoine	0.78	0.87	0.15	0.17	0.0000	0.0001	0.0026	0.0784	0.0001	0.0020	5.1118	0.7893	3.9715	0.6836
12,13-DiHOME	1.91	0.95	0.83	0.41	0.0000	0.0002	0.1684	0.3319	0.0000	0.0000	1.0532	0.8789	2.0159	0.8313
chiro-inositol	0.42	1.45	2.02	7.00	0.0000	0.0003	0.0051	0.0924	0.0001	0.0032	0.8855	1.7864	0.3703	2.5916
3-methoxytyramine sulfate	0.99	0.90	0.82	0.75	0.0000	0.0007	0.0182	0.1476	0.0001	0.0028	1.1757	0.9601	1.1617	0.8685
N2-acetyllysine	0.99	0.93	0.72	0.67	0.0001	0.0013	0.0181	0.1476	0.0002	0.0060	1.3679	0.9894	1.3606	0.9167
hydantoin-5-propionic acid	1.39	1.16	0.56	0.46	0.0001	0.0015	0.0094	0.1250	0.0007	0.0137	0.9711	0.5396	1.3485	0.6263
galactonate	1.16	0.95	1.71	1.40	0.0001	0.0024	0.0003	0.0153	0.0321	0.1065	0.9098	1.5594	1.0526	1.4772
pyrraline	1.04	1.05	0.49	0.49	0.0001	0.0024	0.0032	0.0799	0.0046	0.0431	1.7031	0.8401	1.7747	0.8781
propionylcarnitine	1.18	1.13	0.83	0.80	0.0003	0.0054	0.0107	0.1250	0.0051	0.0462	1.1185	0.9259	1.3164	1.0502
2-ethylhexanoate	0.98	1.04	1.23	1.30	0.0003	0.0054	0.0146	0.1444	0.0037	0.0407	0.9041	1.1110	0.8898	1.1535
1,5-anhydroglucitol (1,5-AG)	1.05	1.01	0.87	0.84	0.0003	0.0049	0.0154	0.1445	0.0025	0.0316	1.0932	0.9513	1.1449	0.9592
1-linolenoylglycerophosphocholine (18:3n3)*	0.84	0.60	0.82	0.59	0.0003	0.0051	0.0408	0.1910	0.0009	0.0175	1.7763	1.4560	1.4847	0.8723
3-(4-hydroxyphenyl)propionate	1.25	2.25	1.87	3.37	0.0004	0.0058	0.0092	0.1250	0.0071	0.0571	0.5569	1.0404	0.6950	2.3401
myristoyl sphingomyelin*	0.97	0.92	0.87	0.81	0.0004	0.0059	0.0361	0.1872	0.0017	0.0268	1.1012	0.9556	1.0733	0.8745
indoleacetylglutamine	0.63	0.78	0.52	0.65	0.0005	0.0066	0.0078	0.1219	0.0108	0.0657	2.4555	1.2850	1.5584	1.0067
3-(4-hydroxyphenyl)lactate	1.08	1.02	0.87	0.82	0.0006	0.0083	0.0251	0.1612	0.0046	0.0431	1.0658	0.9285	1.1533	0.9485
tyrosine	0.95	1.00	0.86	0.90	0.0007	0.0088	0.0036	0.0838	0.0363	0.1140	1.0940	0.9423	1.0418	0.9400
gamma-glutamyltyrosine	0.99	1.04	0.83	0.87	0.0007	0.0088	0.0050	0.0924	0.0272	0.0986	1.0797	0.8980	1.0730	0.9342
palmitoyl-linoleoyl-glycerophosphoinositol (1)*	1.02	0.99	0.73	0.70	0.0008	0.0090	0.0151	0.1445	0.0102	0.0657	1.2822	0.9330	1.3130	0.9198

Appendix table 1. Significant *time effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Time Main Effect		NPJ Final / NPJ Baseline		HPJ Final / HPJ Baseline		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
butyrylcarnitine	2.02	1.42	0.84	0.59	0.0009	0.0103	0.0809	0.2465	0.0019	0.0268	1.1662	0.9800	2.3583	1.3933
biliverdin	0.96	1.00	1.22	1.27	0.0009	0.0103	0.0182	0.1476	0.0109	0.0657	1.1350	1.3796	1.0907	1.3801
cis-urocanate	1.37	1.48	1.84	1.99	0.0011	0.0118	0.0132	0.1383	0.0191	0.0856	0.7886	1.4471	1.0787	2.1448
proline	1.06	1.15	0.85	0.92	0.0011	0.0118	0.0018	0.0664	0.1025	0.2053	1.0495	0.8897	1.1120	1.0264
5-hydroxyindoleacetate	1.05	1.14	1.15	1.25	0.0012	0.0118	0.0242	0.1612	0.0108	0.0657	0.9160	1.0556	0.9634	1.2074
N-formylmethionine	1.04	1.06	1.08	1.11	0.0013	0.0128	0.0244	0.1612	0.0124	0.0679	0.9537	1.0324	0.9875	1.0942
bilirubin (Z,Z)	0.98	1.02	1.25	1.30	0.0014	0.0131	0.0228	0.1612	0.0142	0.0731	1.0598	1.3215	1.0415	1.3544
N-acetyl-aspartyl-glutamate (NAAG)	0.97	0.97	1.13	1.14	0.0017	0.0154	0.0243	0.1612	0.0170	0.0802	0.9654	1.0957	0.9353	1.0635
5alpha-androstan-3beta,17beta-diol disulfate	0.67	0.67	1.22	1.23	0.0018	0.0154	0.0100	0.1250	0.0430	0.1242	1.4971	1.8308	1.0013	1.2354
5alpha-androstan-3beta,17alpha-diol disulfate	1.04	1.19	1.18	1.36	0.0018	0.0154	0.0704	0.2378	0.0055	0.0480	1.2144	1.4379	1.2610	1.7178
oxalate (ethanedioate)	0.93	1.06	1.15	1.31	0.0018	0.0154	0.0674	0.2337	0.0060	0.0504	0.9295	1.0728	0.8650	1.1335
carnitine	0.93	0.95	0.88	0.91	0.0020	0.0165	0.0109	0.1250	0.0463	0.1283	1.0798	0.9518	1.0016	0.9079
epiandrosterone sulfate	0.79	0.93	1.18	1.39	0.0020	0.0164	0.0428	0.1910	0.0114	0.0657	1.3548	1.5976	1.0659	1.4829
3-methoxybenzenepropanoic acid	0.58	2.37	1.14	4.68	0.0022	0.0169	0.1709	0.3319	0.0023	0.0301	1.6875	1.9244	0.9748	4.5616
glucose	1.02	0.94	0.96	0.88	0.0022	0.0169	0.2290	0.3779	0.0015	0.0246	1.0350	0.9913	1.0600	0.9353
O-methylcatechol sulfate	0.74	1.28	1.08	1.88	0.0022	0.0169	0.1949	0.3537	0.0019	0.0268	1.1349	1.2253	0.8358	1.5717
gamma-glutamylphenylalanine	1.06	1.05	0.91	0.91	0.0029	0.0218	0.0397	0.1889	0.0209	0.0875	1.0556	0.9637	1.1206	1.0154
13-HODE + 9-HODE	2.31	1.53	0.75	0.50	0.0031	0.0221	0.1578	0.3281	0.0040	0.0421	1.0493	0.7910	2.4198	1.2085
3-methoxytyrosine	0.95	0.95	1.10	1.10	0.0031	0.0221	0.0300	0.1797	0.0297	0.1005	0.9913	1.0901	0.9444	1.0388
N-acetylglycine	1.12	1.16	1.59	1.64	0.0033	0.0229	0.0386	0.1872	0.0248	0.0946	0.9976	1.5838	1.1150	1.8323
2-aminooctanoate	1.04	0.90	0.72	0.62	0.0035	0.0238	0.0969	0.2520	0.0093	0.0657	1.3668	0.9827	1.4153	0.8796
N-acetyltyrosine	0.93	0.94	0.84	0.86	0.0036	0.0243	0.0421	0.1910	0.0256	0.0946	1.2442	1.0484	1.1540	0.9901
kynurenate	1.10	1.07	0.87	0.85	0.0038	0.0246	0.0713	0.2388	0.0148	0.0736	1.0741	0.9383	1.1866	1.0074
arachidonate (20:4n6)	1.17	1.28	1.13	1.23	0.0039	0.0248	0.0890	0.2490	0.0118	0.0657	0.9173	1.0354	1.0762	1.3279
glycerate	1.11	1.01	1.19	1.08	0.0042	0.0262	0.0046	0.0923	0.1961	0.2672	0.8804	1.0433	0.9751	1.0534
homostachydrine*	0.97	1.17	0.70	0.84	0.0042	0.0262	0.0046	0.0923	0.1975	0.2672	1.1925	0.8311	1.1599	0.9721
4-hydroxyhippurate	0.57	1.62	0.90	2.57	0.0044	0.0265	0.4721	0.4792	0.0010	0.0186	1.7912	1.6181	1.0212	2.6203
myristoleoyl sphingomyelin*	1.20	1.03	0.83	0.71	0.0045	0.0268	0.0962	0.2520	0.0132	0.0698	0.8511	0.7095	1.0243	0.7313

Appendix table 1. Significant *time effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Time Main Effect		NPJ Final / NPJ Baseline		HPJ Final / HPJ Baseline		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
9,10-DiHOME	2.09	1.02	1.04	0.51	0.0047	0.0274	0.7301	0.5526	0.0004	0.0094	1.0375	1.0753	2.1637	1.1007
myo-inositol	1.05	1.01	1.20	1.15	0.0048	0.0279	0.0180	0.1476	0.0804	0.1879	0.9017	1.0799	0.9437	1.0873
dopamine sulfate (1)	0.69	1.03	0.47	0.69	0.0056	0.0315	0.0444	0.1925	0.0410	0.1233	1.9786	0.9243	1.3744	0.9513
acetoacetate	1.39	0.78	2.07	1.16	0.0061	0.0335	0.0047	0.0923	0.2711	0.3042	0.7779	1.6110	1.0830	1.2592
threonate	0.93	0.99	1.19	1.28	0.0061	0.0335	0.0598	0.2207	0.0339	0.1095	0.8950	1.0649	0.8289	1.0576
taurolithocholate 3-sulfate	0.89	0.68	1.90	1.46	0.0063	0.0341	0.0032	0.0799	0.3487	0.3487	1.1368	2.1578	1.0103	1.4771
N-acetylphenylalanine	0.98	1.05	0.87	0.92	0.0066	0.0350	0.0271	0.1676	0.0798	0.1879	1.1110	0.9620	1.0929	1.0097
ribitol	0.99	0.94	0.91	0.86	0.0067	0.0351	0.1453	0.3103	0.0134	0.0699	1.0752	0.9798	1.0626	0.9162
cinnamoylglycine	2.13	2.42	1.19	1.36	0.0074	0.0381	0.5307	0.4889	0.0018	0.0268	1.1289	1.3466	2.3996	3.2590
12-HETE	1.17	6.79	0.90	5.27	0.0082	0.0407	0.1977	0.3567	0.0114	0.0657	2.0041	1.8129	2.3351	12.3061
prolylglycine	0.91	0.94	0.71	0.73	0.0083	0.0407	0.1147	0.2730	0.0238	0.0938	1.4211	1.0043	1.2873	0.9401
palmitoyl-linoleoyl-glycerophosphocholine (1)*	1.07	0.91	0.99	0.85	0.0083	0.0407	0.7511	0.5557	0.0009	0.0175	0.9803	0.9719	1.0459	0.8849
N-acetylkynurenine (2)	0.96	1.27	0.68	0.90	0.0090	0.0433	0.0111	0.1250	0.2209	0.2772	1.3381	0.9094	1.2860	1.1533
dopamine sulfate (2)	0.72	1.03	0.55	0.79	0.0094	0.0447	0.0463	0.1925	0.0733	0.1779	1.7500	0.9626	1.2579	0.9893
dihydroferulic acid	0.74	4.91	0.75	5.00	0.0100	0.0470	0.5962	0.5068	0.0001	0.0035	1.7967	1.3524	1.3290	6.6416
xanthurenate	1.60	2.06	0.71	0.91	0.0104	0.0480	0.0983	0.2520	0.0381	0.1178	0.7920	0.5604	1.2672	1.1525
2-aminophenol sulfate	1.45	0.59	0.99	0.40	0.0106	0.0484	0.4349	0.4523	0.0046	0.0431	1.5037	1.4953	2.1858	0.8785
hexanoylglycine	1.20	1.03	1.44	1.24	0.0112	0.0502	0.0382	0.1872	0.1067	0.2105	0.9298	1.3345	1.1118	1.3807
3-hydroxyhippurate	0.60	0.92	0.91	1.40	0.0115	0.0502	0.5532	0.4977	0.0032	0.0379	1.8730	1.7116	1.1237	1.5745
3-methylglutaconate	1.09	1.06	0.89	0.87	0.0116	0.0502	0.1652	0.3313	0.0235	0.0938	1.1410	1.0189	1.2400	1.0812
taurine	0.95	1.02	1.15	1.25	0.0116	0.0502	0.1000	0.2520	0.0430	0.1242	0.9466	1.0931	0.8970	1.1199
1-arachidonoylglyercophosphate	1.06	1.43	1.29	1.74	0.0141	0.0599	0.1248	0.2884	0.0430	0.1242	1.0743	1.3873	1.1426	1.9880
2-hydroxypalmitate	0.89	0.97	1.05	1.14	0.0142	0.0599	0.4239	0.4523	0.0074	0.0571	1.0326	1.0884	0.9238	1.0532
gentisate	1.97	2.01	1.44	1.47	0.0144	0.0600	0.2796	0.3932	0.0151	0.0736	0.6691	0.9653	1.3190	1.9443
7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	0.98	1.05	0.86	0.92	0.0154	0.0635	0.0250	0.1612	0.2109	0.2731	1.1678	1.0059	1.1449	1.0586
androsterone sulfate	0.70	0.80	1.23	1.40	0.0162	0.0659	0.1037	0.2561	0.0630	0.1575	1.3723	1.6844	0.9590	1.3412
hippurate	0.83	1.20	1.08	1.57	0.0170	0.0672	0.3811	0.4332	0.0117	0.0657	1.3491	1.4628	1.1214	1.7614
oleoyl-linoleoyl-glycerophosphoinositol (1)*	1.00	0.97	0.82	0.79	0.0172	0.0672	0.1307	0.2928	0.0525	0.1402	1.2032	0.9914	1.2078	0.9601

Appendix table 1. Significant *time effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Time Main Effect		NPJ Final / NPJ Baseline		HPJ Final / HPJ Baseline		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
palmitoyl-arachidonoyl-glycerophosphocholine (1)*	1.09	0.92	0.95	0.80	0.0172	0.0672	0.6798	0.5330	0.0037	0.0407	1.0560	1.0053	1.1549	0.9232
taurocholenate sulfate	1.38	1.14	1.36	1.12	0.0173	0.0672	0.0172	0.1476	0.3046	0.3198	1.1043	1.5022	1.5276	1.7117
5alpha-androstan-3alpha,17alpha-diol monosulfate	0.78	0.90	1.13	1.31	0.0176	0.0673	0.2432	0.3806	0.0245	0.0946	1.1661	1.3155	0.9068	1.1895
guanidinosuccinate	1.58	0.95	1.93	1.17	0.0179	0.0675	0.0536	0.2037	0.1344	0.2324	0.5438	1.0508	0.8569	1.0008
methionine sulfone	0.87	1.04	1.20	1.43	0.0182	0.0675	0.4033	0.4426	0.0116	0.0657	0.7535	0.9079	0.6556	0.9403
alpha-CEHC glucuronide*	1.05	0.89	0.72	0.61	0.0183	0.0675	0.2475	0.3806	0.0254	0.0946	1.0174	0.7334	1.0669	0.6554
2,3-dihydroxyisovalerate	1.64	0.74	2.07	0.93	0.0184	0.0675	0.0466	0.1925	0.1559	0.2450	1.3534	2.8000	2.2238	2.0765
3-(3-hydroxyphenyl)propionate	0.74	0.99	1.08	1.44	0.0187	0.0678	0.4978	0.4831	0.0082	0.0622	1.6856	1.8274	1.2487	1.8014
N3-methyluridine	0.95	0.76	0.89	0.71	0.0193	0.0694	0.2329	0.3779	0.0297	0.1005	1.1155	0.9941	1.0546	0.7537
oleate (18:1n9)	1.31	1.02	1.30	1.01	0.0200	0.0703	0.0082	0.1219	0.5237	0.4044	0.7965	1.0361	1.0456	1.0570
alanine	0.93	1.01	0.87	0.95	0.0200	0.0703	0.0229	0.1612	0.2888	0.3145	1.1138	0.9724	1.0365	0.9811
1-linoleoylglycerophosphocholine (18:2n6)	1.08	0.82	1.00	0.76	0.0207	0.0716	0.8418	0.5725	0.0029	0.0353	1.1212	1.1185	1.2077	0.9137
beta-hydroxyisovaleroylcarnitine	1.02	0.92	0.94	0.84	0.0209	0.0716	0.2335	0.3779	0.0329	0.1072	1.0659	0.9972	1.0829	0.9137
2-hydroxyoctanoate	1.37	0.97	0.82	0.58	0.0210	0.0716	0.3333	0.4105	0.0197	0.0864	1.1461	0.9439	1.5736	0.9120
N-acetyl-beta-alanine	1.00	0.99	0.91	0.90	0.0215	0.0724	0.0745	0.2443	0.1226	0.2257	1.1314	1.0268	1.1330	1.0216
3,4-dimethoxy-hydrocinnamic acid	0.56	1.71	0.80	2.43	0.0218	0.0725	0.1529	0.3231	0.0592	0.1535	1.5221	1.2162	0.8585	2.0820
cysteine	1.05	0.92	1.28	1.12	0.0220	0.0725	0.0332	0.1864	0.2437	0.2947	0.8572	1.0942	0.9003	1.0040
phenyllactate (PLA)	1.09	1.06	0.93	0.91	0.0225	0.0732	0.1636	0.3313	0.0569	0.1485	1.1303	1.0501	1.2304	1.1162
valerylcarnitine	1.41	1.42	0.86	0.87	0.0226	0.0732	0.2784	0.3932	0.0286	0.0999	1.0819	0.9352	1.5258	1.3240
valine	1.00	1.05	0.91	0.96	0.0231	0.0741	0.0339	0.1864	0.2524	0.2996	1.0398	0.9454	1.0377	0.9945
2-hydroxydecanoate	1.45	0.95	1.05	0.69	0.0244	0.0774	0.8368	0.5716	0.0038	0.0407	1.0184	1.0686	1.4745	1.0175
stearoyl sphingomyelin	1.01	1.02	1.09	1.10	0.0255	0.0802	0.1750	0.3340	0.0618	0.1564	0.9825	1.0673	0.9917	1.0863
methylsuccinate	1.45	1.08	1.28	0.95	0.0268	0.0837	0.0298	0.1797	0.3184	0.3298	0.8338	1.0693	1.2119	1.1538
mannitol	0.38	1.34	0.26	0.92	0.0274	0.0846	0.0200	0.1581	0.4165	0.3695	4.4159	1.1479	1.6717	1.5431
docosahexaenoate (DHA; 22:6n3)	1.27	1.20	1.20	1.13	0.0276	0.0846	0.0331	0.1864	0.3052	0.3198	0.8652	1.0344	1.0977	1.2397
5-acetylamino-6-formylamino-3-methyluracil	1.21	0.56	1.03	0.47	0.0280	0.0850	0.5927	0.5068	0.0107	0.0657	1.1027	1.1320	1.3396	0.6362
kynurenine	0.99	0.98	0.92	0.91	0.0288	0.0852	0.1414	0.3053	0.0915	0.1974	1.0751	0.9902	1.0645	0.9659
glucarate 1,4-lactone	0.83	1.01	1.11	1.35	0.0290	0.0852	0.3103	0.4054	0.0346	0.1108	1.0265	1.1417	0.8534	1.1539

Appendix table 1. Significant *time effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Time Main Effect		NPJ Final / NPJ Baseline		HPJ Final / HPJ Baseline		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
4-hydroxyphenylpyruvate	0.89	0.88	0.88	0.87	0.0293	0.0852	0.0818	0.2465	0.1604	0.2452	1.2262	1.0733	1.0857	0.9483
cys-gly, oxidized	0.96	1.00	0.86	0.91	0.0294	0.0852	0.0864	0.2484	0.1529	0.2430	1.2242	1.0547	1.1694	1.0585
3-methoxycatechol sulfate (2)	1.16	1.01	0.73	0.64	0.0294	0.0852	0.2559	0.3849	0.0462	0.1283	1.5092	1.0951	1.7457	1.1093
cytidine	0.52	1.02	0.36	0.70	0.0296	0.0852	0.0145	0.1444	0.5311	0.4061	2.8666	1.0255	1.4829	1.0432
10-nonadecenoate (19:1n9)	1.39	1.18	1.26	1.07	0.0316	0.0894	0.0435	0.1910	0.2871	0.3138	0.7908	0.9984	1.1005	1.1772
3-hydroxybutyrate (BHBA)	2.28	1.05	2.11	0.97	0.0316	0.0894	0.0183	0.1476	0.4935	0.3913	0.7731	1.6274	1.7657	1.7105
2-aminoheptanoate	1.16	1.19	0.79	0.82	0.0322	0.0901	0.0916	0.2512	0.1608	0.2452	1.2136	0.9647	1.4027	1.1483
2-hydroxyglutarate	1.03	0.74	1.48	1.05	0.0324	0.0901	0.0340	0.1864	0.3481	0.3487	0.8805	1.2994	0.9086	0.9583
glycolithocholate sulfate*	1.22	0.68	2.03	1.13	0.0327	0.0902	0.0083	0.1219	0.7382	0.4776	1.0193	2.0646	1.2444	1.4123
leucine	1.01	1.04	0.93	0.96	0.0332	0.0902	0.0761	0.2464	0.1953	0.2672	1.0406	0.9654	1.0497	1.0036
3-phenylpropionate (hydrocinnamate)	1.32	1.75	1.14	1.51	0.0333	0.0902	0.8796	0.5793	0.0054	0.0480	1.0045	1.1405	1.3217	1.9980
N-octanoylglycine	1.47	1.20	1.42	1.16	0.0336	0.0903	0.0463	0.1925	0.2914	0.3145	0.7640	1.0836	1.1226	1.2974
deoxycholate	1.56	0.92	1.09	0.64	0.0343	0.0908	0.7969	0.5606	0.0020	0.0274	1.1041	1.2077	1.7210	1.1083
succinylcarnitine	1.13	1.16	0.88	0.90	0.0346	0.0908	0.3067	0.4054	0.0449	0.1276	1.1320	0.9942	1.2828	1.1540
methyl indole-3-acetate	0.84	1.53	0.50	0.92	0.0348	0.0908	0.0468	0.1925	0.2999	0.3185	1.9604	0.9818	1.6439	1.5043
pyridoxate	1.25	0.88	1.37	0.97	0.0351	0.0908	0.0011	0.0417	0.6057	0.4377	0.9287	1.2688	1.1586	1.1192
isovalerylglycine	1.11	1.00	0.82	0.74	0.0351	0.0908	0.1989	0.3573	0.0801	0.1879	1.2270	1.0077	1.3678	1.0118
gamma-glutamyl-2-aminobutyrate	1.11	1.18	1.10	1.17	0.0364	0.0921	0.3405	0.4140	0.0413	0.1233	0.9372	1.0295	1.0369	1.2103
L-urobilin	1.15	1.20	1.58	1.65	0.0368	0.0921	0.1867	0.3497	0.0915	0.1974	0.4735	0.7478	0.5452	0.9006
eicosenoate (20:1n9 or 11)	1.43	1.09	1.31	1.00	0.0373	0.0921	0.0157	0.1445	0.6069	0.4377	0.8425	1.1072	1.2046	1.2055
1,2,3-benzenetriol sulfate (2)	0.11	0.33	0.16	0.48	0.0375	0.0921	0.0950	0.2520	0.1847	0.2643	19.3834	3.1354	2.1544	1.0415
quinolinate	1.10	1.14	0.84	0.87	0.0377	0.0921	0.1338	0.2969	0.1348	0.2324	1.1641	0.9777	1.2829	1.1175
N-acetylserine	1.00	1.05	1.03	1.08	0.0379	0.0921	0.4921	0.4830	0.0240	0.0938	0.9861	1.0125	0.9857	1.0637
2-hydroxyisobutyrate	1.05	1.15	1.04	1.14	0.0381	0.0921	0.4948	0.4830	0.0240	0.0938	0.9592	0.9988	1.0073	1.1471
phenylcarnitine*	0.79	0.85	1.19	1.28	0.0381	0.0921	0.0796	0.2464	0.2183	0.2759	1.1988	1.4290	0.9507	1.2135
gamma-glutamylvaline	1.05	1.12	0.89	0.95	0.0383	0.0921	0.0650	0.2295	0.2580	0.2996	1.0659	0.9437	1.1207	1.0612
histidine	1.01	1.03	0.84	0.86	0.0384	0.0921	0.1632	0.3313	0.1119	0.2160	0.9830	0.8268	0.9882	0.8532
3-hydroxyquinine	1.19	1.00	0.91	0.77	0.0387	0.0923	0.3748	0.4331	0.0390	0.1196	0.5863	0.5340	0.6963	0.5340

Appendix table 1. Significant *time effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Time Main Effect		NPJ Final / NPJ Baseline		HPJ Final / HPJ Baseline		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
citrate	1.13	1.03	1.17	1.07	0.0403	0.0949	0.0621	0.2252	0.2814	0.3106	0.9204	1.0747	1.0367	1.1098
gamma-CEHC	1.04	0.94	0.85	0.77	0.0404	0.0949	0.1840	0.3462	0.1046	0.2076	1.3148	1.1183	1.3706	1.0557
alpha-hydroxycaproate	1.23	1.04	0.94	0.80	0.0411	0.0960	0.4811	0.4825	0.0283	0.0999	1.0007	0.9372	1.2297	0.9789
hydroquinone sulfate	1.47	1.36	0.72	0.67	0.0425	0.0979	0.0952	0.2520	0.2114	0.2731	1.2250	0.8861	1.8022	1.2078
flavin adenine dinucleotide (FAD)	1.35	1.13	0.86	0.71	0.0426	0.0979	0.1732	0.3322	0.1192	0.2217	0.7862	0.6724	1.0641	0.7592
mead acid (20:3n9)	1.38	1.03	1.28	0.95	0.0431	0.0985	0.0133	0.1383	0.7267	0.4759	0.8035	1.0296	1.1124	1.0623
adenosine	0.52	0.60	0.72	0.84	0.0439	0.0994	0.0424	0.1910	0.3984	0.3628	2.6315	1.9023	1.3614	1.1428
N-palmitoyl glycine	0.94	1.08	1.02	1.17	0.0441	0.0994	0.7471	0.5557	0.0129	0.0695	0.9893	1.0042	0.9274	1.0852

Appendix table 2. Significant *treatment effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA		MEDIAN SCALED CONCENTRATIONS			
	HPJ/NPJ		Final/Baseline		Treatment Main Effect		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value				
ethyl glucuronide	0.61	0.41	1.30	0.88	0.0002	0.0984	1.3081	1.7005	0.7944	0.7014

Appendix table 1. Significant *treatment:time interaction effect* metabolites in all overweight and obese adults after 12-wk NPJ or HPJ interventions

Biochemical Name	FOLD CHANGE				ANOVA						MEDIAN SCALED CONCENTRATION			
	HPJ/NPJ		Final/Baseline		Treatment:Time Interaction		Ctrl Final / Ctrl Basal		PEOJ Final / PEOJ Basal		NPJ Baseline	NPJ Final	HPJ Baseline	HPJ Final
	Baseline	Final	NPJ	HPJ	*p*-value	*q*-value	*p*-value	*q*-value	*p*-value	*q*-value				
12,13-DiHOME	1.91	0.95	0.83	0.41	0.0013	0.2774	0.1684	0.3319	0.0000	0.0000	1.0532	0.8789	2.0159	0.8313
dihydroferulic acid	0.74	4.91	0.75	5.00	0.0015	0.2774	0.5962	0.5068	0.0001	0.0035	1.7967	1.3524	1.3290	6.6416
linoleoylcarnitine*	1.69	0.93	1.31	0.72	0.0017	0.2774	0.0477	0.1929	0.0084	0.0623	0.9313	1.2166	1.5748	1.1284

ACADEMIC EDUCATION

Sept 2009 – Dec 2010: Master's Degree in Nutritional, Environmental and Genetic Factors for Growth and Development, University of Granada
Sept 2003-May 2009: Bachelor's Degree in Nutrition and Food Sciences, Universidad Iberoamericana, Puebla, Mexico

PROFESSIONAL EXPERIENCE

Jan 2010 – Present: PhD student at the Group "CTS-461- Nutrition Biochemistry: therapeutic Implications" in Granada, Spain
Mar 2015 – Jun 2015: Visiting PhD student at the Human Performance Lab in the North Carolina Research Campus, in the Appalachian State University North Carolina. Supervisor: Prof. Dr. David Nieman.
May 2010 – Dec 2010: Internship at the group "CTS-461- Nutrition Biochemistry: therapeutic Implications" in Granada, Spain. Supervisor. Prof. Concepción Aguilera.
Sept 2008 - May 2009: Internship in the Nutrition Department, Universidad Iberoamericana, Puebla, Mexico. Supervisor; Prof. Claudia Rodriguez.
Sept 2008 – Dec 2008: Assistant professor, Molecular Biology, Universidad Iberoamericana, Puebla, Mexico. Supervisor: Prof. Beatriz Abundis.

SCIENTIFIC PUBLICATIONS

Rangel-Huerta OD, Aguilera CM, Martin MV, Soto MJ, Rico MC, Vallejo F, Tomas-Barberan F, Pérez-de-la-Cruz A, Gil A, Mesa MD. Normal or high polyphenol content in orange juice affects antioxidant activity, blood pressure, and body weight in obese or overweight adults. J Nutrition, 2015. Accepted. (Forthcoming). doi:10.3945/jn.115.213660

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Pastor-Villaescusa, Belen, Oscar D. Rangel-Huerta, Concepcion M. Aguilera, and Angel Gil. 2015. "A Systematic Review of the Efficacy of Bioactive Compounds in Cardiovascular Disease: Carbohydrates, Active Lipids and Nitrogen Compounds." Annals of Nutrition and Metabolism 66 (2-3): 168–81. doi:10.1159/000430960.

Rangel-Huerta, Oscar D, Concepcion M Aguilera, Maria D Mesa, and Angel Gil. 2012. "Omega-3 Long-Chain Polyunsaturated Fatty Acids Supplementation on Inflammatory Biomakers: A Systematic Review of Randomised Clinical Trials." The British Journal of Nutrition 107 Suppl (June): S159–70. doi:10.1017/S0007114512001559.

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Oscar Daniel Rangel Huerta; Calder, Philip; Angel Gil Hernandez. Ácidos grasos poliinsaturados omega-3 y sistema inmunitario. Libro Blanco de los Omega 3. pp. 231 - 242. 2013.

PARTICIPATION IN RESEARCH PROJECTS

Evaluation of a dairy product of low energetic content and low glycaemic index in obese children with non-alcoholic fatty acid liver disease (PRONAOS-NAFLD)

Evaluation of the glycaemic index of a dairy product and its effect on appetite control (PRONAOS-Indice Glicemico. Puleva Biotech S.A.)

Scientific research for the development of new generation foods for weight management and obesity prevention (CENIT-PRONAOS, CDTI).

Evaluation of a new orange-based beverage enriched with polyphenols (Whole Press) on features of metabolic syndrome and cardiovascular risk factors in overweight and obese adults humans (Coca-Cola Services SA/SN)

STIPENDS AND AWARDS

Programa de Fortalecimiento de las Capacidades en I+D+I de la Consejería de Economía, "Movilidad Internacional de Jóvenes Investigadores de Programas de Doctorado Universidad de Granada y CEI BioTic Granada stipend"

SEMINARS, CONGRESSES, COURSES

III Congreso de la Federación Española de Sociedades de Nutrición, Alimentación y Dietética. 2015. Seville, Spain

III Meeting of Young Researchers in Nutrition. 2015. Sevillle, Spain

XVI Reunión de la Sociedad Española de Nutrición. 2014. Pamplona, Spain.
II Meeting of Young Researchers in Nutrition. 2014. Pamplona, Spain.

XXXVIII Congreso de la Sociedad Española de Ciencias Fisiológicas. 2014. Granada, Spain
VI Seminar on Healthy Diet and Novel Foods "Healthy diet and exercise, health claims and food reformulation" 2013. Granada, Spain

I Meeting of Young Researchers in Nutrition. 2013. Madrid, Spain

IUNS 20th International Congress of Nutrition. 2013. Granada, Spain.

XXVVII Congreso SENPE. 2012. Madrid, Spain.
6th International Immunonutrition Workshop. 2012. Palma de Mallorca, Spain.

61 Congreso de la Asociación Española de Pediatría. 2012. Granada, Spain.

11TH European Nutrition Conference (FENS). 2011. Madrid, Spain

COMMUNICATIONS IN CONGRESSES

Identificación De Metabolitos Asociados Con La Ingesta De Zumo De Naranja. Rangel-Huerta, Oscar Daniel; Aguilera García, Concepción Maria; Perez De La Cruz, Antonio; Gil Hernández, Ángel; Mesa, Maria Dolores. 2015. Oral Communication. III Congreso De La Federación Española De Sociedades De Nutrición, Alimentación Y Dietética.

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El Tratamiento Con Metformina En Niños Obesos Disminuye El Riesgo Cardiovascular**.** XIV Congreso De La Sociedad Española De Investigación En Nutrición Y Alimentación En Pediatría. 2014. Pastor-Villaescusa, María Belén; Rangel-Huerta, Oscar Daniel; Gil-Hernandez, Angel; Aguilera-García, Concepción María

El Tratamiento Con Metformina En La Obesidad Infantil Presenta Mejores Resultados En Niños Prepúberes Respecto A Niños Púberes. XVI Reunión De La Sociedad Española De Nutrición. 2014. Pamplona. Pastor-Villaescusa, María Belén; Rangel-Huerta, Oscar Daniel; Gil-Hernandez, Angel; Aguilera-García, Concepción María

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El Consumo De Un Zumo Con Flavanonas Mejora La Defensa Antioxidante Y Disminuye El Estrés Oxidative. XVI Reunión De La Sociedad Española De Nutrición. Poster. 2014. Rangel-Huerta, Oscar Daniel; Aguilera-García, Concepción María; Tomás-Barberán, Francisco; Perez-De La Cruz, Antonio Jesus; Gil-Hernandez, Angel; Mesa-Garcia, Maria Dolores

Total Body Water As A Possible Marker Of Metabolism Alteration In Obese Children And Adolescents. I International Hydration Congress And III National Hydration Congress. Poster. 2013. Madrid, España. Rangel-Huerta, Oscar Daniel

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Procollagen III N-Terminal Propeptide (PIIINP) As A New Biomarker Of Metabolic Disorders In Childhood Obesity, Beyond A Fibrosis Biomarker At Early Ages. IUNS (International Union Of Nutritional Sciences) 20th International Congress Of Nutrition. Oral Communication. 2013.

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Efecto De Un Zumo De Naranja Enriquecido En Flavanonas Sobre Biomarcadores De Riesgo Cardiometabóllco En Adultos Con Distinto Grado De Obesidad O Sobre Peso. IX Congreso De La Sociedad Española De Nutrición Comunitaria - SENC 2012. Oral Communication. 2012. Cádiz, España. Rangel-Huerta, Oscar Daniel; Gil-Hernandez, Angel; Aguilera-García, Concepción María; Mesa-Garcia, Maria Dolores

Evolution Of Plasma Inflammatory Biomarkers After The Intake Of An Orange-Based Beverage Enriched With Polyphenols In Overweight Adults (BIONAOS Study). 6th International Immunonutrition Workshop. Poster. 2012. Palma De Mallorca, Spain. Rangel-Huerta, Oscar Daniel; Gil-Hernandez, Angel; Mesa-Garcia, Maria Dolores; Aguilera-García, Concepción María

Respuesta De La Resistina Plasmática En Niños Obesos Tras La Ingesta De Un Batido Lácteo De Bajo Índice Glicémico.. XIV Congreso De La Sociedad Española De Nutrición. Poster. 2012. Zaragoza, Spain**.** Rangel-Huerta, Oscar Daniel

Efecto De La Ingesta De Un Batido De Bajo Índice Glícemico Sobre Los Niveles De Hormonas Gastrointestinales Relacionadas Con El Control Del Apetito Y La Secreción De Insulin. XXXIV Congreso National De La SEEP. Poster. 2011. Oral Communication. Cantabria, Santander. Rangel-Huerta, Oscar Daniel