Design and development of meat-based functional foods with walnut: Technological, nutritional and health impact

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Abstract

With growing understanding of the relationship between diet and health has come the emergence of so-called functional foods. The idea of using food for health purposes and not merely as a source of nutrients opens up a whole new field in the meat industry. In addition to traditional presentations, there a number of ways in which the meat sector can modify the qualitative and quantitative composition of meat and meat product components and produce designer foods with specific properties. This entails addressing quality factors associated with different product properties (sensory and technological properties, hygiene, convenience, stability, etc.), nutritional value (balanced composition and bioactive substances) and their effects on physiological function and health. This article reviews a comprehensive model for the development of meat-based functional foods based on a presentation of the research achieved in terms of the design and development of qualitatively and quantitatively modified meat products (through reformulation) in nutrients associated with cardiovascular risk (walnut as a source of bioactive substances). It also discusses their bioavailability and the effect of their consumption on intermediate cardiovascular risk markers in humans.

Key words: Functional food; meat products, walnut, technological development; bioavailability; cardiovascular disease risk

Short title: Walnut-enriched meat as a functional food

1. Introduction

Growing understanding of the relationship between diet and health is leading to new insights into the effect of food ingredients on physiological function and health, inducing increased consumer demand for healthy, nutritious foods with additional health promoting functions, such as functional foods.

Over the last several decades, meat products have come under increasing scrutiny by medical, nutritional and consumer groups because of the associations established between their consumption (or that of a number of their constituents—fat, cholesterol, etc.) and the risk of some of the major degenerative and chronic diseases (ischemic heart disease, cancer, hypertension and obesity). Therefore, meat-based functional foods are being seen as an opportunity to improve the "image" of meat and address consumer needs, and also to update the nutritional and dietary goals (Jiménez-Colmenero, 2007a).

Most research into meat-based functional foods has been founded on animal production or technological strategies to increase the presence of healthy compounds (Arihara, 2006; Jiménez-Colmenero, 2007b; Jiménez-Colmenero, Carballo, & Cofrades, 2001; Muguerza, Gimeno, Ansorena, & Astiasarán, 2004). This article reports a comprehensive approach to the design and development of reformulated meat-based functional foods in which animal fat is reduced and bioactive compounds (walnut) are added in appropriate amounts to achieve a functional effect. This functional effect was assessed by means of an intervention study with volunteers presenting increased risk of cardiovascular disease.

2. Dietary intake and cardiovascular diseases (CVD)

Diet and nutrition are important factors in the promotion and maintenance of health throughout life. According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), several dietary patterns along with lifestyle habits constitute major modifiable risk factors in relation to the development of non-communicable diseases, coronary heart disease (CHD), cancer, type 2 diabetes, obesity, osteoporosis and periodontal disease (WHO, 2003). In addition to providing nutrients to cover metabolic requirements, diet can modify specific physiological functions and reduce the risk of certain diseases. Chronic diseases contribute to approximately 60% of deaths, almost half of which are cardiovascular (WHO, 2003). Moreover, deaths and disabilities due to CHD and strokes can be cut by more than 50% by a combination of simple and effective low-cost national efforts and individual actions to reduce major risk factors such as high blood pressure, high cholesterol, obesity and smoking (WHO, 2007).

According to the WHO (2009), by 2010 CVD will be the leading cause of death in developing countries, as these are no longer only diseases of the developed world. The rise in CVD reflects a significant change in dietary habits, physical activity levels, and tobacco consumption worldwide as a result of industrialization, urbanization, economic development and food market globalization. For instance, people nowadays consume a more energy-dense, nutrient-poor diet and are less physically active. Imbalanced nutrition, reduced physical activity and increased tobacco consumption are the key lifestyle factors. High blood pressure, high blood cholesterol, overweight and obesity—and type 2 diabetes—are among the major biological risk factors in CVD. Unhealthy dietary practices include high consumption of saturated fats, salt and refined carbohydrates and low fruit and vegetable intake (WHO, 2003; WHO, 2009), whereas recommendations concerning the reduction of CVD risk in industrialized countries commonly specify less fat (total, saturated and ω-6 polyunsaturated—PUFAs—fatty acids and non-*trans*) (WCRF/AICR, 1997; WHO, 2003).

3. Functional foods.

Observations associating particular eating habits, mainly excessive intake of certain nutrients, with the etiology and development of chronic diseases have led to the concept of "optimal nutrition". Optimal nutrition is based on a number of dietary recommendations to modify (reduce or increase) the intake of certain foods or food components and the development of new foods in which the original composition is modified, in terms of both nutrient and non-nutrient contents. The ultimate aim is to optimize physiological functions and so maximize their contribution to well-being and health and minimize the risk of diseases. It is in this context that functional foods come to the fore. A food may be regarded as functional if it is satisfactorily demonstrated that it beneficially affects one or more target functions in the body, over and above adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or a reduction of the risk of disease. A functional food can be a natural food, or a food to or from which a component has been added or removed by technological or biotechnological means (Diplock, et al., 1999). The incorporation of these products into the diet is highly desirable and could have considerable public health implications, not only because it can reach broad population groups but also because these diseases start at early ages and because they entail a high cost to society and governments in financial terms and in terms of disability-adjusted life years.

The criteria required to assess the scientific basis of claims regarding food properties were laid down in the course of the projects FUFOSE (Diplock et al., 1999) and PASSCLAIM (Aggett et al., 2005; Asp et al., 2004). Finally, a consensus was arrived at on the scientific criteria that need to be followed for purposes of assessment or design of foods for which healthy properties are claimed (Aggett et al., 2005). Changes in composition are not enough for a food to be considered functional; it must also be satisfactorily shown that consumption has a beneficial effect on certain population groups. Trials are therefore necessary to demonstrate such an effect, preferably in humans and using suitable intermediate biomarkers for early detection and prognosis of the disease (Aggett et al., 2005).

Where it is not possible to directly measure the effect of a food in terms of health, quality of life or reduced risk of disease—as in most cases concerning chronic diseases—functionality is assessed by means of biomarkers. In the field of nutrition, biomarkers should be associated with a future health objective, but at a stage where dietary intervention can effectively assist early diagnosis or improved prognosis of the disease in question. Such markers of intermediate objectives within the process of the disease must be carefully selected to allow for short-term measurements that can be used later on to make inferences regarding the effects on final objectives which would only normally be possible in a long-term study.

4. Walnut and cardiovascular disease: Bioactive components

Epidemiological studies show that regular consumption of nuts in general, and walnut in particular (Banel & Hu, 2009; Feldman, 2002; Fitó et al., 2007; Fraser, Sabaté, Beeson, & Strahan, 1992; Iwamoto et al., 2000; Nus, Ruperto, & Sánchez-Muniz, 2004; Sabaté, 1993; Sabate et al., 1993; Salas-Salvadó, García-Arellano et al., 2008; Salas-Salvadó, Fernández-Ballart, 2008; Tyrovolas & Panagiotakos, 2009) correlates inversely with myocardial infarction and CHD regardless of other factors associated with risk such as age, sex, smoking, hypertension, weight and exercise. Although the exact mechanism is not understood, the positive effects of walnut intake have been attributed, at least in part, to its particular lipid composition, which is characterized by a high fat content (62-68 % dry matter) and abundant monounsaturated fatty acids (MUFAs, oleic acid 18 % of total fatty acids) and PUFAs (linoleic and α-linolenic making up 58 and 12 %, respectively of total fatty acids). In addition to these, there are other components of interest: fibre (5-10 % dry matter), protein (14 % dry matter) rich in arginine, vitamins, minerals, phytosterols, polyphenols, etc. (Nus et al., 2004; Ravai, 1995; Sabaté, 1993; USDA, 2005).

Walnuts have been selected as a potential functional component in meat products because their nutritional composition has more relevance to CVD than other nuts and plant foods (e.g. ω-6 and ω-3 PUFAs, γ-tocopherol, arginine-rich protein) and because they are generally acceptable to consumers. In addition, this approach supplies several other constituents of walnuts (e.g. plant sterols, polyphenols and fibre) without substantially reducing other nutritionally important components of meat such as iron and zinc (Olmedilla-Alonso, Granado-Lorencio, Herrero-Barbudo, & Blanco-Navarro, 2006). The amount of walnuts to be incorporated into the final product is partially based on nut and meat consumption data in Spain (INE, 1994; MAPA, 2003), data from epidemiological and clinical studies with nuts, and suggestions about nut intake (FDA, 2003).

In response to the evidence for the beneficial health effect of walnut consumption, stress has recently been placed on the importance of including them as a regular part of the diet. In this context the Food and Drug Administration (FDA) has approved the possibility of a qualified health claim in relation to heart disease. In particular it is permitted to state that "supportive but not conclusive research shows that eating 1.5 ounces (42.5 g) of walnuts, as part of a low saturated fat and low cholesterol diet and not resulting in increased caloric intake, may reduce the risk of coronary heart disease" (FDA, 2003; FDA, 2004). That claim statement on walnuts and heart disease has been reviewed without changes for whole or chopped walnuts (FDA, 2009). For its part, the WHO (WHO, 2003) recommends consuming at least 400 g of fruit and vegetables daily, at least 30 g of which should be nuts, pulses or seeds. In its global strategy programmes for CVD prevention, the WHO (2009) recommends, among other dietary strategies, consuming a diet rich in fruit, vegetables, nuts and whole grains.

Despite such notable advantages, walnut consumption generally falls short of recommended levels. Not many people can be persuaded to systematically consume enough walnuts in their pure state over long periods of time (every day over a long period). They are more likely to accept dishes prepared with it. One way to promote walnut intake would be to use it as an ingredient in frequently-consumed foods, for instance meat derivatives, to which bioactive compounds can be added to render them more heart-healthy (Jiménez Colmenero et al., 2001; Sánchez-Muniz, 2004).

5. Meat products as functional foods

Meat and meat products are essential parts of the diet which concentrate and supply a large number of nutrients (protein, fat, vitamins, minerals). Meat is a fundamental source of proteins of high biological value. It is a well-balanced source of amino acids that satisfies human physiological requirements. Meat is a good source of iron, zinc and phosphorus, with significant amounts of other essential trace elements such as selenium, magnesium and cobalt, and an excellent source of B group vitamins like B_1, B_2, niacin, pantothenic acid, vitamin B_6 and vitamin B_{12} (Jiménez-Colmenero, 2007a). Meat has traditionally been highly appreciated as a food of great nutritional value, and meat consumption has always been associated with good health and prosperity. However, over the last few years the situation has changed, among other reasons because of the epidemiological associations discovered between meat and meat derivatives or some of their constituents, and the risk of some of the major diseases in our society (CHD, cancer, high blood pressure and obesity). Like other food-related sectors, the meat industry is in a permanent state of change in response to continuous technological innovations and changes in consumer demands, among which those relating to improvement of certain health-related aspects through the diet are becoming increasingly important. This situation is prompting the emergence of new, "healthier" meat products, prominent among them functional foods, which are the main driving force behind the development of new food products, including meat-based products. Functional meat derivatives present an excellent opportunity to diversify and take up positions in a tremendously important emerging market (Jiménez-Colmenero, Reig, & Toldrá, 2006).

There are various strategies for introducing qualitative and/or quantitative modifications in meat and meat derivatives in order to achieve a "functional" product, and of these, strategies associated with meat processing are especially promising. The principal advantage of meat derivatives in terms of modifying composition is the opportunity that they offer to change the ingredients (meat and non-meat) used to produce them and hence work with various endogenous and exogenous bioactive compounds. The basic idea is to be able to limit the concentration of compounds with adverse physiological effects and enhance the concentration of other, beneficial ones (Jiménez Colmenero et al., 2001; Jiménez-Colmenero, 2007a; Sánchez-Muniz, 2004). Most physiologically active substances come from plants, and when combined with other foods such as meat derivatives they can help endow a food with functional effects. The idea of using plant products in the meat industry is not a new one; various types of ingredients have been used for their technological, sensory, economic and nutritional effects. Because of their importance, lipids are among the bioactive components that have received most attention, particularly (in quantitative and qualitative terms) with respect to the development of potential meat-based functional foods (Jiménez-Colmenero, 2007b). In that respect, the special characteristics of walnuts offer promising perspectives for the development of functional meat products. As one of the most important commonly-consumed foods, meat offers excellent ways to promote intake of functional ingredients without any radical changes in eating habits.

6. Design of meat-based functional foods with walnuts

Within this context and considering the present state of and trends in meat consumption, our team has embarked on the design and development of walnut-enriched functional meat-based products with potential for CHD risk reduction. Within a multidisciplinary project, we address the development of a functional meat product of this type using reformulation technology. Specifically, our aim is to design and develop meat products qualitatively and quantitatively modified in such a way as to achieve a nutrient composition profile associated with a reduced risk of CHD (Olmedilla-Alonso et al., 2006).

The proposed design incorporates some of the strategies that are known to be effective in preventing CHD (WHO, 2009), such as: limiting energy intake from total fats and shifting fat consumption away from saturated fats to unsaturated fats and towards the elimination of trans-fatty acids; increasing consumption of ω-3 PUFAs from fish oil or plant sources; consuming a diet rich in fruits, vegetables, nuts and whole grains, and low in refined grains; avoiding excess consumption of food with high content of salt and refined carbohydrates. The dietary intake of fats, and especially their quality, strongly influences the risk of CVD like coronary heart disease and stroke, through effects on blood lipids, thrombosis, blood pressure, arterial function, arrhythmogenesis and inflammation. Excess salt has a significant impact on blood pressure levels (WHO, 2009).

For this purpose, several aspects had to be addressed: how much meat could be supplied to be acceptable and sustainable within a balanced diet while being compatible with nutritional recommendations based on the available scientific evidence, which nut(s) and what amounts should and could be incorporated into the final meat product (technological challenge); and, finally, how to assess the efficacy of the product for the purpose for which it had been developed (human intervention trial and use of biomarkers). Walnuts were selected as a good source of nutrients relevant to CVD (e.g. ω-6 and ω-3 PUFAs, γ-tocopherol, arginine-rich protein) compared to other nuts and plant foods. The amount of walnuts and meat to be consumed was based on nut and meat consumption data in Spain (INE, 1994; MAPA, 2003) and recommendations about nut intake (e.g. FDA, 2003; Olmedilla-Alonso et al., 2006). It was thought necessary to formulate at least two types of walnut-enriched meat products (for reasons of diversification), which could reasonably be consumed by a large percentage of the population five times a week as part of a normal diet and sustained over the long term. The study was designed to last five weeks, time enough to be sure of a change in lipid profile following the dietary intervention.

The meat-based functional products had to contain a high concentration of walnut, and therefore they were designed so that 150 g of any of them would supply ca. 70 % of the daily walnut intake recommended to help reduce the risk of cardiovascular diseases (FDA, 2003). That is equivalent to consuming 19.4 g walnut/day. This proposal required a reformulation of meat derivatives involving compositional changes that affect protein quality, lipid content and profile, and the presence of antioxidants.

The approach to the technological and nutritional challenges and physiological testing used in the study reported below may be fully applicable to the development of other functional food products.

7. Technological development of meat products with added walnut

One fundamental aspect of this research is the technological development of meat products formulated with walnut, which must achieve comparable quality levels (sensory, nutritional, technological, health, convenience, etc.) to those of any other meat product of similar characteristics. This also includes other aspects (social, legal, etc.) unrelated to the intrinsic quality of the product which affect their valuation and degree of acceptance. In accordance with the design, the manufacturing process was aimed at both limiting concentrations of certain unwanted compounds (animal fat, sodium) and promoting the presence of bioactive compounds (present in the added walnut) with potentially beneficial effects on the onset and development of cardiovascular disorders.

Based on strategies of this kind, two types of product were developed with clearly different characteristics: *restructured steaks* and *frankfurters* (gel/emulsion systems). These kinds of products were chosen for their special interest. Restructured steaks belong to a range of foods which offer major advantages in industrial terms and can readily satisfy all consumer requirements (appearance, composition, texture, constant quality, convenience, etc.). Then frankfurters belong to a group of derivatives of major economic importance which are widely accepted throughout the world.

The technological approach, manufacturing and preparation procedures, physicochemical and sensory characteristics, storage stability and cooking behaviour of the new derivatives were evaluated. Following is a brief account of the studies carried out, highlighting the impact of various variables on the physicochemical and sensory characteristics of the products as influenced by various factors assayed in the technological manufacturing processes.

7.1 Restructured steaks

One essential aspect of this research was analysis of the influence of the percentage of added walnut (0, 5, 10 and 20 %) on the characteristics of the protein matrix (physicochemical properties, microstructure) and sensory attributes of restructured steaks (Cofrades et al., 2004a; Jiménez-Colmenero et al. 2003; Serrano, Cofrades, & Jiménez-Colmenero, 2004). Although adding walnut produces certain changes in the matrix, the resulting products presented acceptable physicochemical and sensory characteristics. One of the conditioning factors analysed was the effect of the degree of structural disintegration of the meat on the influence of added walnut on product characteristics (Cofrades et al., 2004a). Restructured steaks were formulated with walnut using thermal gelation and cold gelation with microbial transglutaminase. Thermal gelation was used to evaluate the consequences of changing the formulation for purposes of marketing as a frozen product (Jiménez Colmenero et al., 2003) or a pre-cooked product (Cofrades et al., 2004b). Cold gelation procedures were used to make steaks containing different concentrations of walnut (0, 10 and 20%) for chilled storage (Serrano et al., 2004).

In order to improve the outward appearance of the restructured steaks, a number of protein coatings were assayed. The idea was, once the steak was made, to coat it with a protein film that would lend it the appearance of a conventional steak. The development of the product, the application and technology of the coating preparation and various other aspects reported in this article are all protected by patent (Spanish Patent Application Nº 200300367; Spanish Patent Application Nº 200400548).

The reformulation of meat products to enhance health-beneficial components, as in the case of added walnut, produces qualitative and quantitative changes in the composition (protein, lipids and others components) of meat products (Serrano et al. 2005). Some of these changes can influence the product's response to different technological treatments, and hence its chilling and frozen stability. Studies on chilling storage with special emphasis on microbiological aspects and the formation of biogenic amines (Ruiz-Capillas, Cofrades, Serrano, & Jiménez-Colmenero, 2004), and on frozen storage with special emphasis on lipid oxidation (Serrano, Cofrades, & Jiménez-Colmenero, 2006) have shown that the presence of walnut is not a limiting factor for product stability.

Restructured beef steak, like other foods, will normally be cooked prior to consumption. Heat treatment has a significant impact on the composition and physicochemical characteristics of final food products; indeed, it is well known that meat product composition (fat content, added non-meat ingredients) and cooking techniques are among the factors most affecting the final quality attributes of the product. The influence of various cooking methods—grilling, conventional oven, microwave oven and pan-frying—on the composition and physicochemical characteristics of restructured beef steaks has been investigated by Serrano, Librelotto, Cofrades, Sánchez-Muniz and Jiménez-Colmenero (2007). No limitations were observed due to different cooking procedures (roasting, grilling and pan-frying) on the physicochemical characteristics of restructured steaks containing walnut. Despite their high polyunsaturated fat content, lipid oxidation of restructured beef steak containing walnut was low after all cooking procedures. The high lipid retention of walnut restructured steaks during cooking assures a healthy food profile at the moment of ingestion (Serrano et al., 2007). More specific studies have been conducted on the effect of pan-frying in extra virgin olive oil on the fatty acid profile and fatty acid retention and thermal oxidation of restructured functional steaks (Librelotto et al., 2008) and on long frozen storage stability of the same product (Librelotto, Bastida, Zulin-Botega, Jiménez-Colmenero, & Sánchez-Muniz, 2009). This culinary technique produced fried steaks with low lipid alteration (oxidative and hydrolytic). Moreover, the atherogenic and thrombogenic indices of these pan-fried restructured steaks were much lower than those of reduced- or medium-fat counterparts, so that the meat was highly acceptable from a nutritional and a CVD point of view (Librelotto et al., 2009).

7.2 Frankfurters

Alongside the research on restructured steaks, several studies have been conducted to address the technological challenge also posed by reformulation (design and development) of meat products based on gel/emulsion systems. These have assessed how the presence of different amounts of walnut (7, 14 and 21%) influences the characteristics (physicochemical, morphological and sensory) of meat emulsion products, in this case frankfurters. Morphology, textural parameters (hardness, cohesiveness and chewiness), fat- and water-binding properties and colour were all influenced by the level of walnut addition. Generally speaking, products with added walnut have presented sensory attributes acceptable to consumers (Ayo, Solas, Carballo, & Jiménez-Colmenero, 2004; Carballo, Ayo, & Jiménez-Colmenero, 2003). In order to limit sodium levels, various studies have been conducted to assess the effect of several salt levels on products containing walnut (Ayo, Jiménez-Colmenero, Carballo, & Ruiz-Capillas, 2004) and analyse the use of transglutaminase in meat batters with different percentages of walnut and without salt (Cofrades et al., 2004b). On the basis of these results a study was conducted to compare products (with added walnut and no salt) formulated with combinations of transglutaminase and a number of non-meat ingredients (caseinate, KCl and wheat fibre) as salt replacers, with a product containing normal salt levels. The results suggest that some of the combinations assayed could be used to compensate for the absence of salt in the preparation of sausages containing walnut (Jiménez-Colmenero, Ayo, & Carballo, 2005).

We would note in a general way that the quality attributes of the meat products developed—restructured steaks and frankfurters—containing 20 % of walnut were acceptable in terms of physicochemical and sensory properties and stability.

8. Nutritional profile of meat products with added walnut.

The potential functional effect of walnut in meat-based functional foods derives from various constituents with health implications. Studies have been conducted both on restructured steaks (Serrano et al., 2005) and on frankfurters (Ayo et al**.,** 2007) to determine how the walnut affects the nutritional profile. Compared with control products (0 % added walnut), the product with added walnut (20-25 %) presented a lower lysine/arginine ratio, larger quantities of MUFAs and ω-3 PUFAs (mainly α-linolenic acid), a lower ω-6/ω-3 PUFA ratio and a higher PUFA/SFA ratio. In restructured beef steak (13 % fat) formulated with 20 % added walnut, around 90 % of the fat content came from walnut; MUFAs and PUFAs together accounted for almost 90 % of total fatty acids, with ω-6/ω-3 PUFA < 4 and PUFA/SFA > 6.5 (Table 1). The addition of 20 % walnut also furnished around 1 % of dietary fibre. Energy content was 99 kcal/100g (414.2 kJ/100g) in the control sample (1.6 % fat content, no walnut added) and 213 kcal/100 g (891.2 kJ/100g) in the walnut (20 %) enriched product (14.5 % fat content), approximately 62 % of energy from fat. Replacement of raw meat material by walnut reduced the cholesterol content and increased (more than 400 fold) the amount of γ-tocopherol. Meat products with added walnut could be good sources of manganese, iron, copper, potassium and magnesium, supplying high percentages (15-40 %) of the daily recommended intakes for these minerals.

The γ-tocopherol content of restructured meat products is of particular interest as the exposure biomarker chosen in the intervention study to assess the functionality of the products designed—walnut-enriched steaks containing over 4000 µg γ-tocopherol/100g (Table 1), walnut-enriched sausages containing 4980 µg γ-tocopherol/100g and walnut-free steaks containing ca. 19 µg γ-tocopherol/100g (Olmedilla-Alonso, Granado-Lorencio, Herrero-Barbudo, Blanco-Navarro, & Sánchez-Muniz, 2005; Olmedilla-Alonso et al., 2006).

9. Assessing the effect of meat-based functional products on cardiovascular disease risk.

A 5-week randomized, placebo-controlled crossover study was conducted on subjects at risk of CVD (n=25) to assess the functional effect of consumption of restructured steaks and frankfurters containing 20 % walnut. For five weeks, instead of meat and meat derivates the volunteers consumed five meat products (4 steaks and 1 sausage, with or without added walnut) per week (150 g meat product/ helping). The habitual consumption of a mixed diet (no avoidance of any groups) was required. In terms of equivalent walnut consumption, that would be 30 g walnut/steak and 16 g walnut/helping of sausages. This means consuming 136 g of walnuts per week, giving an average intake of 19.4 g walnuts/day, which is ca. 70 % of the amount of walnuts suggested by the (FDA, 2004; FDA, 2009).

Men and women were selected who had at least four CVD risk factors as identified by the WHO/FAO in their report on prevention of chronic diseases through diet (WHO, 2003). Inclusion criteria were: age (men: 45-65, women: 50-70 years and postmenopausal), overweight (BMI > 25 and < 34.90 kg/m^2), serum cholesterol > 220 and < 290 mg/dl, and at least one of the following features: smoking habit and/or blood pressure $\geq 140/90$ mm Hg.

The bioavailability of walnut components contained in the new products was assessed beforehand by means of a postprandial (single-dose) study in three volunteers using γ-tocopherol as an exposure marker. The reason for choosing this compound was that consumption of the meat product with added walnuts supplies significantly more γ-tocopherol (ca. 29 mg γ-tocopherol per week) than is normally supplied by our diet (Olmedilla-Alonso et al., 2005). Consumption of a restructured meat product caused an increase in γ-tocopherol in the triglyceride-rich lipoprotein fraction during the postprandial period only when walnuts were added. The increase peaked 6 hours after intake (time evaluated: 6 h). This provides *in vivo* evidence of the efficacy of walnut-enriched restructured meat products as a vehicle for bioactive substances, e.g. γ-tocopherol (Olmedilla-Alonso et al., 2008).

The functional effects were assessed using clinicaly relevant and related biomarkers of CHD: 1) biomarkers of function: a) used in clinical practice: cholesterol (total, LDL-cholesterol and HDL-cholesterol), triacylglycerols, blood pressure and homocysteine; b) biomarkers sensitive to dietary changes and associated with CVD risk: folic acid, vitamins B_6 and B_{12}, α-tocopherol, serum γ-tocopherol, and platelet function test; c) other biomarkers: eicosanoids (e.g. Thromboxan A_2, prostacyclin I_2, leukotrien B_4), Lp(a), apolipoprotein (Apo) A1, Apo B, platelet aggregation, inflammation markers (PCR and adhesion of monocytes VCAM and ICAM), lipid peroxidation (oxidized LDL, LPO), antioxidant status based on the activity of catalase (CAT), superoxide dismutase (SOD), total glutathione, reduced glutathione (GSH) and oxidized glutathione (GSSG) in red cells and the activities of paraoxonase (PON-1), aryilesterase in plasma; 2) biomarkers of CHD/CVD risk: total cholesterol, LDL-cholesterol and systolic and diastolic blood pressure.

Although several markers have been proposed to study the relationship between diet and CVD, (Aggett et al., 2005; Contor & Asp, 2004; Mensink et al., 2003), only LDL-cholesterol and blood pressure are well-established markers generally accepted as related to changes in risk of CVD, although they are not direct measures of CHD incidence. Diet-related changes in cholesterol and blood pressure could allow claims for enhanced function and would also support disease risk reduction claims.

The effect of the consumption of restructured meat products with and without added walnuts was evaluated by means of the above mentioned biomarkers of function and CHD-related risk. Table 2 shows the ones most commonly used in clinical practice (along with γ-tocopherol as exposure biomarker). On comparing concentrations after the diet containing walnut-enriched meat products with the baseline (habitual mixed diet), there was a decrease in total cholesterol (10.7 mg/dl), LDL-cholesterol (7.6 mg/dl) and body weight (0.5 kg) and a slight decrease in systolic pressure, as well as an increase in γ-tocopherol (8.9 μg/dl). Other biomarkers such as homocysteine, folate and vitamins B_6 and B_{12} remained within normal ranges, as did platelet function (measured as obturation time) (Olmedilla-Alonso et al., 2008)

HDL-cholesterol, fasting triacylglycerols and plasma homocysteine are established examples of markers sensitive to dietary factors and have been validated methodologically, but it is not yet clear to what extent changes in these markers reflect improved state of health and well being and performanceand reduction of disease risk. For haemostatic function and oxidative damage, there is a need to develop and validate markers of improved state of health and disease risk reduction that are sensitive to dietary changes (Aggett et al., 2005).

In this study we expected no changes in body weight, as the dietary intervention only involved the substitution of meat sources in the diet. However, three volunteers out of 25 registered small but significant reductions in body weight with the consumption of walnut-enriched meat with respect to that of the low-fat restructured meat. Weight loss has also been reported in other studies likewise involving a control group and adjusting for energy (Iwamoto et al., 2002), and in a recent prospective study in which increased nut consumption was not associated with greater body weight gain during eight years of follow-up in healthy middle-aged women; instead, it was associated with a slightly lower risk of weight gain and obesity (Bes-Rastrollo et al., 2009).

Studies have been conducted comparing the effect of consumption of walnut-enriched restructured steak and frankfurter with that of consumption of low-fat meat products on different markers of antioxidant status (Canales et al. , 2007) and of platelet aggregation and thrombogenesis (Canales et al., 2009). This intervention study was the first to evaluate the effect on those parameters of the intake of meat products with added walnut in subjects at high risk of developing CHD. Erythrocyte catalase (CAT), superoxide dismutase (SOD), total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG), lipid peroxidation (LPO), and serum uric acid and paraoxonase-1 (PON-1) were all tested in increased-CHD-risk individuals consuming meat products with added walnut with respect to the same individuals consuming control products.

Present data clearly show that the intake, 5 times per week, of meat products with added walnut for 5 weeks increased concentrations/activities of several antioxidant defence biomarkers, such as CAT, SOD, PON1, total glutathione, GSH and GSSG in those volunteers (Canales et al., 2007) (Figures 1 and 2). Natural defence against ROS involves a number of enzymatic and non enzymatic antioxidant mechanisms (Sies, 1991). The particular composition of walnuts (and thus, of walnut enriched meat), which are rich in antioxidant compounds such as vitamin E, α-tocopherol, γ-tocopherol, δ-tocopherol, folic acid and vitamin C (Nus et al., 2004; Olmedilla-Alonso et al., 2006; Ravai, 1995; USDA, 2005), seems to be responsible for improving the antioxidant status of study participants.

The intake of meat products with added walnut increased PON-1 activity irrespective of HDL-C levels, which were not affected by the treatment. This corroborates results of other studies (Almario, Vonghavaravat, Wong, & Kasim-Karakas, 2001). The increase in PON-1 concentrations is presumably a consequence of the intake of a relatively large amount of PUFAs, counterbalanced by the consumption of nut antioxidants. Many other authors have investigated the influence of diet on PON-1 activity, but available results are controversial. The consumption of meat products with added walnut induced a reduction of ~34% LPO concentration in erythrocytes as compared to approximately ~8% in the control products. These results suggest that lipid peroxidation is not increased by meat products with added walnut consumption despite their higher PUFA content. Haque et al. (2003) found that an aqueous extract of walnut reduced the LPO content in liver and kidney of mice induced by chemotherapy toxicity.

Platelet aggregation, plasma thromboxane (TX) A_2 (measured as TXB_2), prostacyclin I_2 (measured as 6-keto-$PGF_1\alpha$) production and the TXB_2/6-keto-$PF_1\alpha$ ratio were determined at baseline and at wk 3 and 5 for the two dietary periods (Canales et al., 2009). As indicated, the diet including meat products with added walnut contained lower SFA and higher PUFA concentrations and presented a more favourable ω-6/ω-3 ratio than the diet including control meat products, which accounts for the larger variations in maximum aggregation values observed at week 5 [9.62 ± 0.67 to 8.28 ± 0.56 cm/5 min ($p<0.05$) in walnut enriched meat *versus* 10.35 ± 0.52 to 10.30 ± 0.66 cm/5 min ($p>0.05$) in control meat] (Canales et al., 2009). The lower linoleic to linolenic acid ratio of the diet including products with added walnut, together with other walnut compounds, should affect platelet reaction capacity (Chan et al., 1993). At week 5, significant differences ($P<0.05$) between treatments were found for maximum aggregation rate, TXB_2 values and the TXB_2/6-keto-$PF_1\alpha$ ratio. The effects on TXB_2 and the TXB_2/6-keto-$PF_1\alpha$ ratio were time-course dependent (Canales et al., 2009).

Platelet aggregation is modulated by the production of TXA_2 and PGI_2. An optimal balance of TXA_2/PGI_2 may be important in the prevention of thrombotic conditions. The diet including walnut-enriched meat products contains a lower concentration of SFA and presents linoleic/linolenic and ω-6/ω-3 PUFA ratios that are 2.5 times lower than those of the diet including non-walnut-enriched meat products, which would account, at least partially, for the results. Proanthocyanidins, naturally occurring plant metabolites commonly found in fruits, vegetables, nuts, seeds, flowers and bark (Bagchi et al., 1997; Fine, 2000), form part of a specific group of polyphenolic compounds called flavonoids (Bravo, 1998). These compounds are reported to have anti-inflammatory and vasodilatory properties (Bagchi et al., 1997; Bagchi et al., 1998), to inhibit lipid peroxidation, platelet aggregation and capillary permeability and to affect, among others, the phospholipase A_2, cyclooxigenase and lipoxygenase enzyme systems (Bagchi et al., 1997; Bagchi et al., 1998; Murray & Pizzorno, 1999; Robert et al., 1990). Moreover, it has been suggested that tocopherol may reduce thrombogenesis (Robert et al., 1990; Szczeklik, Grylewski, Domagala, Dworski, Basista, 1985).

However, the inter-individual variability of these findings was also striking (Canales et al., 2009; Nus et al., 2007), and genetic factors may influence the results. It was therefore hypothesized that volunteers at increased CHD risk having different polymorphisms in CVD gene candidates respond differently to the intake of restructured beef steaks and sausages containing walnuts in terms of platelet aggregation, TXA_2, PGI_2 and the thrombogenic ratio (TXA_2/PGI_2), and antioxidant status (e.g. erythrocyte CAT, SOD, total glutathione, GSH and GSSG). To this end the population that was defined was screened for polymorphisms for APOA4 (Canales et al., 2010) and PON-1 genes (unpublished data). Although modifications in dietary cholesterol and saturated fat affect plasma lipids differently in carriers of each *APOA4* variant (Herron, Lofgren, Adiconis, Ordovás, & Fernández, 2006; Hubacek et al., 2007**),** to date there have been no studies on the effect of an *APOA4* polymorphism on thrombogenesis. Moreover, no data are available on the effect of *APOA4* polymorphism and the varied response to walnut consumption in individuals at increased CVD risk.

PON-1, a HDL-bound enzyme, may be involved in lipoprotein-phospholipid metabolism and may also inhibit lipid peroxide generation in LDL (Canales & Sánchez-Muniz, 2003; Mackness et al., 1998; Nus, Sánchez-Muniz, & Sánchez-Montero, 2006). Available data suggest that PON-1 is likely to be more active in the absence of antioxidants. PON-1 exists in 2 major polymorphic forms which include the replacement of glutamine (Q) by arginine (R) at position 192 (Adkins, Gan, Mody, & La Du, 1993) and of leucine (L) by methionine (M) at position 55 (Garin et al., 1997). It has been postulated that the ability of HDL to protect LDL against peroxidation *in vitro* is significantly lower in HDL particles from PON-1-R192 (Aviram et al., 2000) than in PON-1-Q192 carriers.

Although the results have not yet been fully evaluated and discussed, the data suggest that the decrease of TXB_2 levels and the TXB_2/6-keto-$PGF_1\alpha$ ratio in APOA4-2 with respect to APOA4-1 carriers after the 5-wk treatment was significantly greater in the diet including walnut-enriched products than in the diet including non-walnut-enriched counterparts. Also, our results revealed a different response of antioxidant status in meat products (walnut-enriched vs. non-walnut-enriched) depending on PON-1 polymorphisms. Unpublished data suggest that the changes in antioxidant status markers at week 5 in meat products with added walnut with respect to the control were greater in PON-1(QQ)192 polymorphism carriers than in PON-1 (QR+RR)192 carriers (data not shown).

10. Concluding remarks

In functional foods, if in addition to achieving quality attributes comparable to those of any other product we wish to assure the presence, absence or reduction of a nutrient or other substance that we know to produce a potential beneficial effect—which has to be established with generally accepted scientific data—then design and development are essential steps.

The design and development of potentially functional meat products based on the addition of walnut has been addressed as part of a project whose goals range from the design and development of meat products in which nutrients associated with CVD risk are qualitatively and quantitatively modified to the study of bioavailability and the effect of consumption on intermediate markers of CVD risk in humans. By using technological strategies in the reformulation of meat matrixes, it has been possible to achieve products with a specific composition (less animal fat and sodium and fortified with various bioactive compounds) which present acceptable quality attributes in terms of physicochemical and sensory properties and stability.

Our assessment of the effect of meat-based functional products on subjects at CVD risk suggests that since regular consumption of meat products containing walnuts produces (i) a reduction in intermediate clinical markers of CHD (such as total and LDL cholesterol), (ii) an improvement in antioxidant status and (iii) a reduction in thrombogenesis markers, they may be considered products that meet the requirements of functional foods for subjects at high risk of CVD like the ones included in the present study. As the walnut was used whole, the observed effects may be associated with its peculiar blend of nutrient and phytochemical compounds but cannot be related to any individual ingredient.

Genotyping studies in ample populations should be performed in order to ascertain the actual target subjects for this functional meat.

In view of the observed benefits accruing from the consumption of walnut-enriched meat, meat industries should seriously consider making and marketing meat products of this kind, and consumers should not hesitate to consume them. Nonetheless, more studies are still desirable to test the effect of long-term consumption of this kind of meat products.

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