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4. Nutritional genomics. A new approach in nutrition research

Carlota Oleaga¹, Carlos J. Ciudad¹, Verónica Noé¹
and Maria Izquierdo-Pulido²

¹Department of Biochemistry and Molecular Biology and ²Department of Nutrition and Food Science
School of Pharmacy, University of Barcelona (UB), Spain

Abstract. There is an increasing evidence that nutritional genomics represents a promise to improve public health. This goal will be reached by highlighting the mechanisms through which diet can reduce the risk of common polygenic diseases. Nutritional genomics applies high throughput functional genomic technologies and molecular tools in nutrition research, allowing a more precise and accurate knowledge of nutrient-genome interactions in both health and disease. Understanding the inter-relationships among genes, genes products, and dietary habits is fundamental to identify those who will benefit the most or be placed at risk by nutritional interventions. This chapter provides an overview of this novel nutritional approach, including the most relevant results of our recent research on the nutrigenomic effects of food polyphenols on cancer cells. Those studies would highlight the molecular mechanisms underlying the chemopreventive effects of those bioactive food compounds.

Introduction

Until recently, nutrition research concentrated on nutrient deficiencies and impairment of health. The importance of diet to sustain health,

Correspondence/Reprint request: Dr. Maria Izquierdo-Pulido, Department of Nutrition and Food Science
University of Barcelona, Av. Joan XXIII 08028 Barcelona, Spain. E-mail: maria_izquierdo@ub.edu

prevention and treatment of diseases has been known for a long time. The advent of genomics –high-throughput technologies for the generation, processing, and application of scientific information about the composition and functions of genomes – has created unprecedented opportunities for increasing our understanding of how nutrients modulate gene and protein expression influencing cellular and organismal metabolism and thus, ultimately impacting human health and well-being. Notably, the knowledge of the human genome has dramatically broadened the scope of studies in nutrition science [1-4].

Nutritional genomics is a relatively new and very fast-moving field of research and combines molecular biology, genetics, and nutrition [3, 5]. It provides a genetic understanding for how diet, nutrients or other food components affect the balance between health and disease by altering the expression and/or structure of an individual's genetic makeup. The conceptual basis for this new branch of genomic research is built on the following premises [1,6]:

- Diet and dietary components can alter the risk of disease development by modulating multiple processes involved with the onset, incidence, progression, and/or severity;
- Diet and dietary components can act on the human genome, either directly or indirectly, to alter the expression of genes and gene products.
- Diet and dietary components could potentially compensate for or accentuate effects of genetic polymorphisms.

The term nutritional genomics is frequently used as an umbrella term for two research specialties: **nutrigenomics** and **nutrigenetics**. However, it is important to note the difference between the terms nutrigenomics and nutrigenetics because although these terms are closely related they are not interchangeable. Nutrigenomics focuses on the effects of nutrients on genes, proteins, and metabolic processes, whereas nutrigenetics involves determining the effect of individual genetic variation on the interaction between diet and disease [2,7]. Thus, those working in nutrigenomics investigate the role of nutrients in gene expression, and those working in nutrigenetics determine how genetic polymorphisms (mutations) affect responses to nutrients [7,8]. Moreover, when reviewing scientific literature, other terms appear, such as epigenetics, transcriptomics, proteomics or metabolomics. All of them describe processes, new tools or situations of this emerging field of nutrition (Table 1). The key challenge is to determine

whether it is possible to utilize this information meaningfully to provide reliable and predictable personalized dietary recommendations for specific health outcomes.

Nutrigenetics and nutrigenomics hold much promise for providing better nutritional advice to the general public, genetic subgroups and individuals [11]. In the future, the integration of nutrition and genomics may lead to the enhanced use of personalized diets to prevent or delay the onset of disease and to optimize and maintain human health. The objectives of this chapter are to provide an overview of this novel nutritional approach. Moreover, we will also include the most relevant results of our research on the nutrigenomic effects of food polyphenols on cancer cells. In addition to the essential nutrients, such as calcium, zinc, selenium or vitamins, there are a variety of classes of nonessential nutrients and bioactive components, such as polyphenols, that seem to significantly influence health. Those bioactive components are known to modify a number of cellular processes associated with health and disease prevention, including carcinogen metabolism, hormonal balance, cell signaling, cell cycle control, apoptosis, and angiogenesis. Our studies are focused in highlighting the molecular mechanisms underlying the chemopreventive effects of those bioactive food compounds.

Table 1. Definitions of terms used in nutritional genomics [9,10].

Term	Definition
Nutrigenomics	Investigates the effects of nutrients and other food components on genes, proteins, and metabolic processes. Transcriptomics, proteomics and metabolomics are used in nutrigenomics research
Nutrigenetics	Investigates the effect of individual genetic variation on the interaction between diet and disease. Genomics are often used in nutrigenetics studies
Epigenetics	Investigates the genome modifications that are copied from a generation to another but not implying changes on DNA sequence
Transcriptomics	Investigates gene expression changes at the mRNA level in response to different stimuli. Utilizes variety of technologies, most commonly microarrays and next-generation sequencing
Proteomics	Analyses all the proteins in a biological system, their interactions and their functional states although effectively, usually only the most abundant subset of 300 or so proteins is relatively easily analyzed
Metabolomics	Investigates the metabolome that consists of all of non-proteinaceous, small molecules present in a biological system. Changes in the metabolome content reflect the biological responses to external stimuli (nutrients among others), which involves altered gene expression and protein production/ activity associated with metabolic pathways

1. Nutrigenetics

Nutrigenetics focuses on the effects that genetic variations have on the binomial diet/disease or on the nutritional requirements and recommended intakes for individuals and populations. To achieve its objectives, the methodology used in nutrigenetics includes the identification and characterization of genetic variants that are associated with, or are the responsible for a different response to certain nutrients or food components [6,11]. These variations generically designated as polymorphisms, including the polymorphisms of a single nucleotide (SNP, single-nucleotide polymorphisms), differences in the number of copies, inserts, deletions, duplications and rearrangements or reorganizations. Undoubtedly, SNPs are the most frequent as they appear every 1,000 base pairs [12].

These differences may determine the susceptibility of an individual to have a disease related to diet or to one or some diet components, as well as to influence in the individual's response to diet changes. There is certain parallelism between nutrigenetics and pharmacogenetics, although in the field of nutrition is more difficult to draw conclusions, since there are important differences between drugs and food components, such as chemical purity, number of therapeutic targets and duration of the exposure, among others [3, 9, 11].

One of the best-described examples of the effect of SNPs is the relationship between folate and the gene encoding for MTHFR (5,10-methylenetetrahydrofolate reductase) [13]. MTHFR has a role in supplying 5-methylenetetrahydrofolate, which is necessary for the re-methylation of homocysteine to form methionine. Methionine is essential to many metabolic pathways including production of neurotransmitters and regulation of gene expression. Folate is essential to the efficient functioning of this MTHFR. There is a common polymorphism in the gene for MTHFR that leads to two forms of protein: the wild type (C), which functions normally, and the thermal-labile version (T), which has a significantly reduced activity. People with two copies of the wild-type gene (CC) or one copy of each (CT) appear to have normal folate metabolism. Those with two copies of the unstable version (TT) and low folate accumulate homocysteine and have less methionine, which increases their risk of vascular disease and premature cognitive decline [14].

Thus, in people with low folic acid intake, higher serum homocysteine levels would be detected in TT homozygotes compared with other genotypes, which would lead them to an increased risk of cardiovascular disease (Figure 1). However, when the intake of folic acid in diet is higher, this increased

amount would compensate the DNA defect in people with the TT polymorphism, and homocysteine serum concentrations would not reach such high values and consequently not show hyperhomocysteinemia. According to this example of gene-diet interaction, a practical application for cardiovascular disease prevention would be to recommend a higher daily consumption of folic acid-rich food to those people with the TT genotype, since these individuals have higher folic acid requirements than the general population due to their genetic susceptibility.

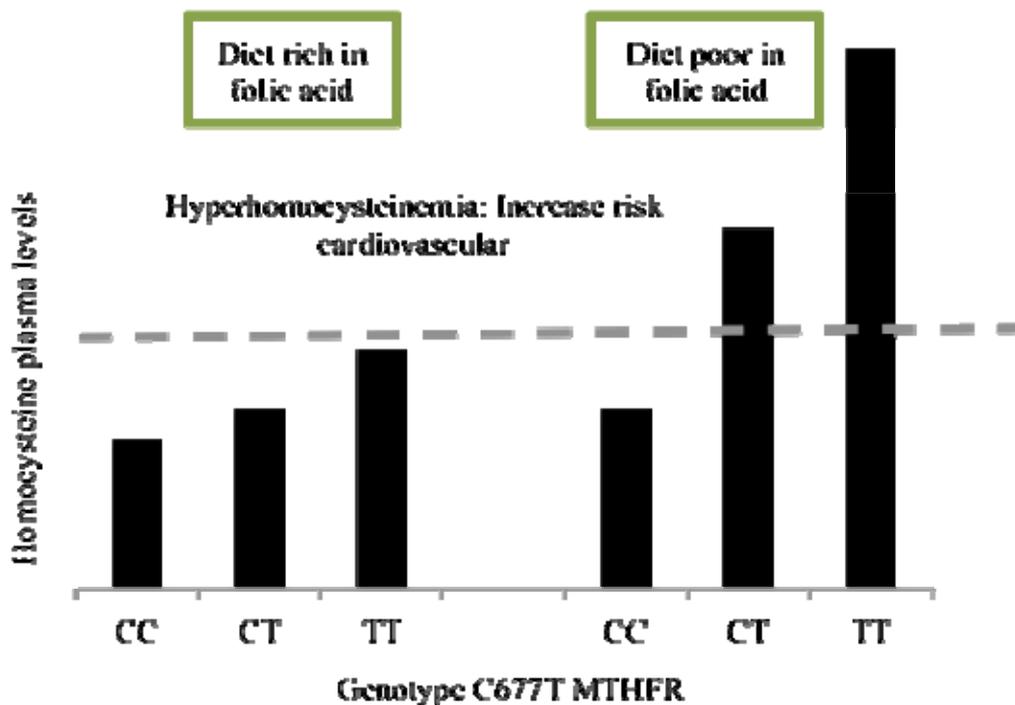


Figure 1. Gene-diet interaction. Folic acid intake may modulate the genetic risk of hyperhomocysteinemia conferred by the C677T polymorphism in the MTHFR gene. Hyperhomocysteinemia only would happen when the mutation occurs with a low folate intake [Adapted from 15].

Another of the genes on which a very active research has been developed is the one that encodes for the synthesis of the lipoprotein APOA1 [16]. APOA1 is the main component of plasmatic HDL and seems to play an important role in the transport of cholesterol. It has been reported that a polymorphism in the gene promoter the -75 A/G (substitution of guanine by adenine), has an influence on the individual's response to polyunsaturated fatty acids (PUFA) intake. Thus, women with the A/A genotype showed higher HDL-cholesterol levels in plasma after ingestion of PUFA, whereas those with genotypes A/G and G/G (wild type) did not show HDL-cholesterol

changes or even a certain decrease in response to the PUFA from diet (Figure 2). Therefore, for the individuals with the genotype A/A the ingestion of PUFA could be a good diet recommendation since it increases HDL. Those results illustrate the complexity of polymorphism-phenotype associations and underscore the importance of accounting for interactions between genes and environmental factors in population genetic studies.

The examples cited here and many others that can be found in the literature published until now [10,11,17-20] illustrate perfectly why nutrigenetics is also termed personalized nutrition, since its major goal is to identify and characterize genes, and nucleotide variants within these, that are associated (or account for) the differential responses to nutrients. In addition to providing a more rational basis for giving personalized dietary advice, the knowledge gained by applying genomic information to nutrition research will also improve the quality of evidence used for making population-based dietary recommendations. The sequencing of an individual's genome has fueled interest in the field of personalized medicine [21,22], but replicating and validating nutrigenetic studies need to remain a priority before personalized nutrition can be considered a worthwhile approach to improve human health [23].

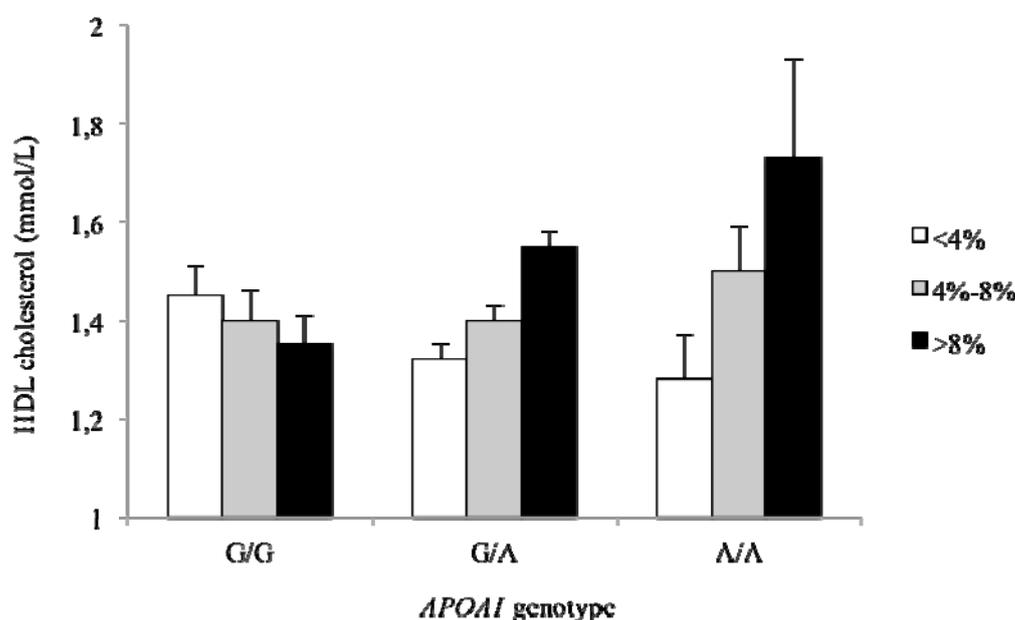


Figure 2. Effect of polyunsaturated fatty acid intake (>4%, 4-8% and >8% of energy) on high-density lipoprotein (HDL) cholesterol blood levels in women. Means were adjusted for age, body mass index, alcohol consumption, tobacco smoking, and intakes of energy, saturated fatty acids, monounsaturated fatty acids, and PUFAs [Adapted from 16].

2. Nutrigenomics

The term nutrigenomics was coined ten years ago to describe a branch of nutrition and food research that applies new profiling techniques for transcripts, proteins and metabolites to better understand the interplay of the genome with its nutritional environment. In this respect, nutrigenomics is still in its infancy and it will need time until it really delivers what was originally hoped [3,6,9].

The field of nutrigenomics harnesses multiple disciplines and includes dietary effects on genome stability (DNA damage at the molecular and chromosome level), epigenome alterations (DNA methylation), RNA and micro-RNA expression (transcriptomics), protein expression (proteomics) and metabolite changes (metabolomics), all of which can be studied independently or in an integrated manner [11, 24]. In this approach, nutrients, other food components, and even whole diets, are considered as “dietary signals” that are detected by “cellular sensors”. These sensors, that are part of cellular signaling cascades, can affect, in turn, all the processes involved in cell function. Therefore, they influence the transcription, translation and protein expression and different metabolic pathways, which ultimately form the phenotype [25, 26].

Using the current genomic tools that include transcriptomics, proteomics and metabolomics, there are two approaches in nutrigenomic research. The first would identify genes, proteins or metabolites that are affected by the diet (nutrients or bioactive compounds) and determine which are the mechanisms involved in this interaction and, consequently, figure out the regulation pathways through which the diet induces these changes. In the second approach, early biomarkers are sought (genes, proteins or metabolites) that are linked with certain dietary compounds or to the whole diet [1,24]. Those biomarkers could act as a “warning signals” about changes in the homeostasis with could have implications for the health [10,11,24].

There are numerous examples [9,11,27,28] that illustrate the interaction between food components and the genome, from mammalian cells in culture to human studies. However, most applications are still of descriptive nature. As an example of a typical nutrigenomic approach research, we will explain our research which its main goal is to study mechanisms underlying the potential chemopreventive effects of a certain type of well-known food compounds called polyphenols.

Polyphenols are the most abundant antioxidants in the diet. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, cocoa, chocolate, and dry legumes also contribute to the total polyphenol intake. Their total dietary intake could

be as high as 1g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants [29]. Despite their wide distribution in plants, the health effects of dietary polyphenols have come to the attention of nutritionists only rather recently. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus [30]. However, our knowledge still appears too limited to formulate recommendations for the general population or for particular populations at risk of specific diseases.

For many years, polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mechanism of action [31,32]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [33]. Both antioxidant and prooxidant effects of polyphenols have been described, with contrasting effects on the cell's physiologic processes. As antioxidants, polyphenols may improve cell survival; as prooxidants, they may induce apoptosis and prevent tumor growth [30, 32]. However, the biological effects of polyphenols may extend well beyond the modulation of oxidative stress. One of the best-known examples involves the interaction of soy isoflavones with estrogen receptors and the effects of these compounds on endocrine function. These effects could explain the prevention by isoflavones of bone resorption among postmenopausal women [30]. A detailed understanding of the molecular events underlying these various biological effects is essential for the evaluation of the overall impact on disease risk and progression.

2.1. Coffee polyphenols and breast cancer: A transcriptomics approach

Coffee is one of the most popular and widely consumed beverages throughout the world. Recent meta-analyses demonstrate inverse associations between coffee intake and the risk of colon, liver, breast and endometrial cancer [34-37]. In prospective population-based cohort studies, the inverse association between coffee consumption and risk of cancer has also been showed. The group of Naganuma *et al.* [38] found that the consumption of at least one cup of coffee per day was associated with a 49% lower risk of upper gastrointestinal cancer in a Japanese population, while Wilson *et al.* [39] found that men who regularly drink coffee appeared to have a lower risk of developing a lethal form of prostate cancer. The lower risk was evident when consuming either regular or decaffeinated coffee.

It has been proposed that the inverse association between coffee intake and colon cancer could be explained, at least in part, by the presence of phenolic compounds in coffee [40]. Among the different phenolic compounds in coffee, the most abundant are hydroxycinnamic acids, which exist mainly in the esterified form. The best example is chlorogenic acid (5-caffeoylquinic acid). In fact, coffee is the major source of chlorogenic acid in the human diet; the daily intake in coffee drinkers ranges from 0.5 to 1 g, whereas coffee abstainers will usually ingest <100 mg/day. Studies have showed that approximately the 33% of ingested chlorogenic acid and 95% of caffeic acid are absorbed intestinally [41]. Thus, about two-thirds of ingested chlorogenic acid reaches the colon where it is probably metabolized to caffeic acid [42]. Bioavailability data suggest that the biological effects of chlorogenic acid would become apparent after its metabolism to caffeic acid, and hence studying the effects of this acid is necessary.

As mentioned before, there is enough evidence from epidemiological data supporting the theory that coffee seems to reduce the risk of certain types of cancer; however, the molecular mechanisms underlying the chemopreventive effects of coffee remain unknown. Using a transcriptomics approach, the effect at the molecular level of the main phenolic compound in coffee, caffeic acid, at concentrations equivalent to one cup of coffee on human colon cancer cells (HT29) was studied. Furthermore, the effect of coffee polyphenols was also evaluated in breast cancer cells.

Colon adenocarcinoma HT29 cells were incubated with caffeic acid at a concentration equivalent to one cup of coffee for 24 hours. It was previously determined that this concentration did not cause any cytotoxic effect in the cell incubations. Then, gene expression was analysed by hybridization to the GeneChip Human Genome U133A plus 2.0 microarrays from Affymetrix, containing 47,000 transcripts and variants. Quantification was carried out with GeneSpring GX v.11.5.1 software (Agilent Technologies), which allows multi-filter comparisons using data from different experiments to perform the normalization, generation of lists and the functional classification of the differentially expressed genes.

A list of differentially expressed genes by 1.3-fold with a p-value cut-off of <0.05 was generated. Upon incubation with caffeic acid, 12 genes were overexpressed whereas 32 genes were underexpressed. Among the overexpressed genes, 33% belonged to the Transcription factors category, 25% to Cell cycle, and 17% to Biosynthetic processes or Immune response. Within the underexpressed genes, again the category corresponding to Cell cycle was the most affected (30% of the genes) followed by Biosynthetic processes (15%) and Transcription factors (12%). Using these data, a Biological Association Network (BAN) was constructed using the Pathway

Analysis within the GeneSpring v.11.5.1, as described in Selga *et al.* [43]. Signal transducer and activator of transcription 5B (STAT5B) and Activating transcription factor 2 (ATF-2) appeared as highly interconnected nodes (Figure 3). In fact, STAT5B was overexpressed with respect to the control by 33,4% in cells treated with caffeic acid, whereas ATF-2 was found underexpressed in HT29 incubated with caffeic acid (26% decrease compared to the control).

The changes in mRNA expression of these two main (STAT5B and ATF-2) nodes were confirmed by RT-PCR and at protein level by Western blot analysis (Figure 4). The key function of STAT5B is to mediate



Figure 3. Biological Association Network (BAN) of differentially expressed genes under caffeic treatment. The BAN was constructed with the Pathway Analysis software within GeneSpring v11.5.1. An expanded network was constructed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation. Only proteins are represented. The BAN shows the node genes STAT5B and ATF-2 that were further studied.

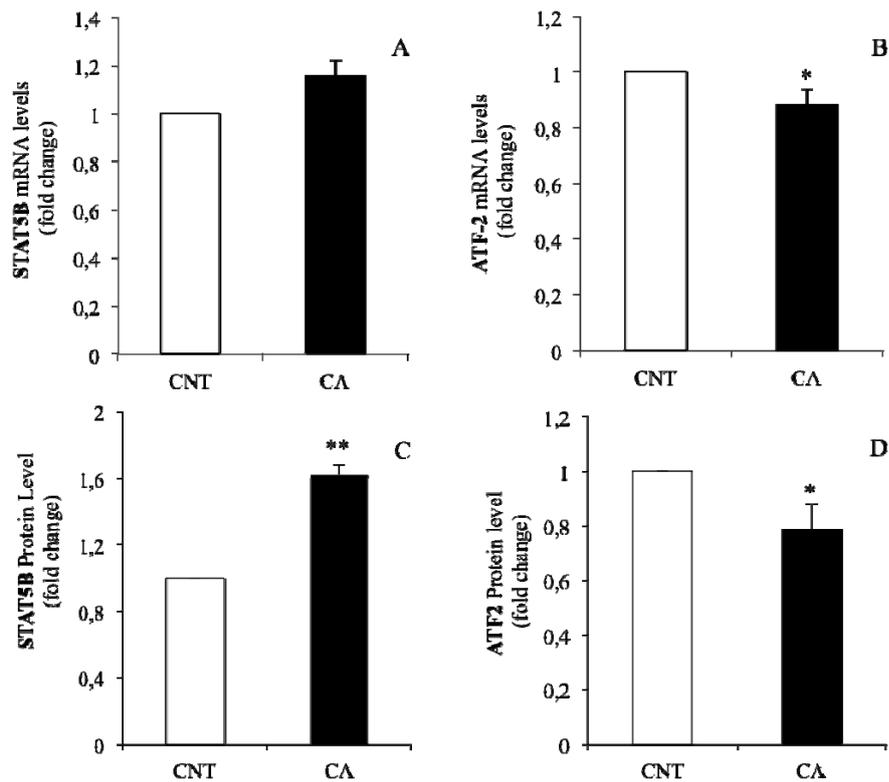


Figure 4. Quantification of mRNA and protein levels for STAT5B and ATF-2 in HT29 cells. The mRNA levels of STAT5B (A) and ATF-2 (B) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) by RT-Real Time. Results are expressed in fold-changes compared to the control, and are the mean + SE of 3 different experiments. * $p < 0.05$ compared with the corresponding control. The protein levels of STAT5B (C) and ATF-2 (D) were determined in control HT29 cells (empty bars) and cells treated with caffeic acid (CA, filled bars) by Western blot. Blots were reprobated with an antibody against β -actin or tubulin to normalize the results. Results represent the mean \pm SE of 3 different experiments. * $p < 0.05$ and ** $p < 0.01$ compared with the corresponding control.

the effects of the Growth Hormone, as STAT5B-null mice failed to respond effectively to this hormone [44]. Modulation of STAT5 levels or transcriptional activity has already been described in cells treated with natural compounds such as nobiletin, acitrus flavonoid [45] thea flavins [46] and silibinin, a natural polyphenolic flavonoid which is a major bioactive component of silymarin isolated from *Silybum marianum* [47]. Activation of STAT5A/B in human breast cancer has been shown to positively correlate with the differentiation status of the tumour. STAT5 have been also shown to transcriptionally regulate E2-sensitive proliferative genes such as cyclin D1 and c-Myc [48] suggesting that STAT5 may play a role in E2-stimulated breast cancer growth. STAT5 activation has also been linked to regulating the expression of the cell cycle control protein cyclin D1 both directly and indirectly [48-50].

On the other hand, ATF-2 is a member of the ATF-cAMP response element-binding protein (CREB) family of transcription factors that can bind to the cAMP response element (CRE) found in many mammalian gene promoters [51]. ATF-2 exhibits both oncogenic and tumor suppressor functions [52]. CREs are found in several genes involved in the control of the cell cycle, e.g., the cyclin D1 gene and ATF-2 binding to this sequence stimulates the transcription of cyclin D1 [53]. ATF-2 has been correlated with proliferation, invasion, migration, and resistance to DNA-damaging agents in breast cancer cell lines.

Therefore, the two main nodes identified in our work regulate cyclin D1 transcription. Cyclin D1 is an important regulator of G1-S phase transition, and its expression in breast cancer cells is sensitive to estrogens and antiestrogens [54]. Cyclin D1 is over expressed at the mRNA and protein level in over 50% of the breast cancers either in the presence or absence of gene amplification and it is one of the most commonly over expressed proteins in breast cancer [55]. In order to know the influence that caffeic acid could have over cyclin D1 levels, since the expression of STAT5B and ATF-2 is modified by this phenolic compound, cyclin D1 levels in MCF-7 cells were analyzed upon incubation with caffeic acid by Western Blot. As shown in Figure 5, incubation of MCF-7 cells with caffeic acid led to a drastic decrease in the levels of cyclin D1 protein, together with an increase in the levels of STAT5B, but there was no decrease in the levels of ATF-2.

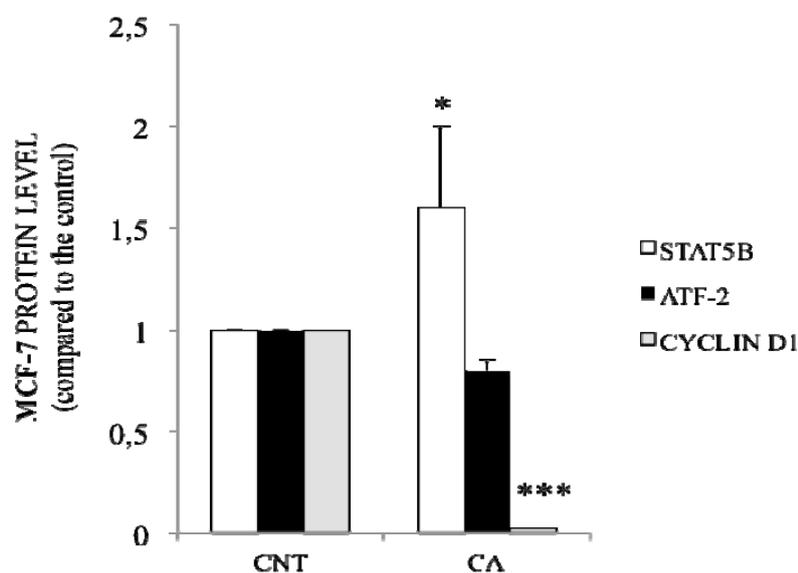


Figure 5. Expression of cyclin D1 upon incubation with caffeic acid in MCF-7 cells. The protein levels were determined in control MCF-7 cells (CNT) and in cells treated with caffeic acid (CA) by Western blot. Blots were reprobated with an antibody against β -actin to normalize the results. Results represent the mean \pm SE of 3 different experiments. * $p < 0.05$ and *** $p < 0.001$ compared with the corresponding control.

It is believed that compounds that modulate cyclin D1 expression could have a role in the prevention and treatment of human neoplasia. For instance, flavopiridol, a synthetic flavonoid based on an extract from an Indian plant used for the potential treatment of cancer, induces a rapid decline in cyclin D1 steady-state protein levels [54]. Taking all these results together, inhibition of cyclin D1 expression appears to be a good approach for cancer treatment. In this direction our observation that coffee and caffeic acid are able to drastically reduce the expression of cyclin D1 in breast cancer cells could suggest that some coffee components could be used as a coadjuvant therapeutic tool in the treatment of breast cancer.

2.2. Cocoa polyphenols and changes in the CYP1A1 gene expression

Cocoa is rich in polyphenols. In fact, cocoa has the highest flavanol contents of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids [56]. The main subclasses of flavonoids found in cocoa are flavanols, particularly the flavanol monomers catechin and epicatechin, and their oligomers, also known as procyanidins [57]. Many examples of the health benefits of cocoa consumption can be found in the literature [58].

Epidemiologic studies of cocoa intake and cancer risk are few, and those assessing overall mortality provide only weak support of the benefits of cocoa. However, human intervention trials indicate that cocoa favours intermediary factors in cancer progression—specifically, markers of antioxidant status [59]. Moreover, there is growing evidence that polyphenols may play a role in regulating apoptosis [60]. Apoptosis may be triggered intrinsically, through the mitochondrial pathway or extrinsically by death ligands and receptors. It is the external pathway that may potentially be modulated by bioactive food components. Flavanols found in cocoa have exhibited pro-apoptotic effects. Proanthocyanadins inhibited growth of human lung cancer cells *in vitro* and *in vivo* [61], and epicatechin synergistically enhanced apoptosis in lung cancer cells treated with epigallocatechin-3-gallate (EGCG) [62]. Cocoa polyphenols have also been found to inhibit the mutagenic activity of heterocyclic amines *in vitro* and *ex vivo* [63].

It has been reported that catechins from green tea could be effective in modulating estrogen-induced breast carcinogenesis, either interfering with receptor mediated pathways or reducing the production of genotoxic estrogen metabolites [64,65]. In our functional genomic study, we sought to evaluate the effect of cocoa flavonoids in a type of breast cancer cells (MCF-7), that are estrogen-receptor (ER)- dependent [66]. Estrogens are implicated in the initiation and promotion stages of breast cancer, and lifetime estrogen

exposure is a major risk factor for breast cancer [67]. Estrogens exert their carcinogenic effects by both estrogen receptor (ER)-dependent and independent mechanisms [68]. Most human breast cancers are initially positive for ER, and their growth can be stimulated by estrogens and inhibited by antiestrogens such as tamoxifen.

For that purpose, MCF-7 cells were incubated for 24h with a purified polyphenol cocoa extract (PCE). PCE was used as representative of the wide flavonoid spectrum (monomers and oligomers) present in cocoa and the concentrations used were not toxic. The differential gene expression analysis was done using PCR arrays. In particular, the expression profile of the 84 genes included in the Stress & Toxicity PathwayFinder™ PCR Array was analyzed in MCF-7 cells both control and treated with a PCE. It was observed that the exposure to PCE decreased the expression of serpine 1 and up-regulated the expression of the CYP1A1, GADD45A, GDF15, GPX1, RAD23A, TP53, and XRCC2 genes (Table 2).

Among those genes, CYP1A1 was chosen for further validation since: (a) it was one of the most overexpressed gene upon incubation with PCE, (b) its overexpression in response to polyphenols had already been described, and (c) it plays an important role in the oxidative metabolism of estrogens. CYP1A1 is a candidate gene for low-penetrance breast cancer susceptibility because it plays an important role in the metabolism of xenobiotics or carcinogens as well as in the oxidative metabolism of estrogens [2004]. CYP1A1 encodes aryl hydrocarbon hydroxylase (AHH) which catalyzes a

Table 2. List of under- and overexpressed genes in MCF-7 cells upon incubation with PCE for 24hours¹.

MCF-7 Gene symbol	Fold-up or down- regulation	p-value
	Test sample / control sample	
CYP1A1	17.60	0.0001
GADD45A	4.20	0.0264
GDF15	2.60	0.0001
GPX1	4.25	0.0183
RAD23A	13.90	0.0394
SERPINE1	- 49.90	0.0216
TP53	2.26	0.0470
XRCC2	17.50	0.0356

¹The expression of each gene was reported as the fold change obtained after each treatment relative to control after normalization of the data. A cut-off of 2-fold was chosen since small changes in gene expression may represent important changes downstream those differentially expressed genes. Lists of differentially expressed genes, with a p-value<0.05, were generated from three independent experiments.

hydroxylation reaction in Phase I metabolism as a first step to increase the polarity of different molecules. Some of these metabolites can be more active than the initial molecules and behave as electrophilic compounds, thus initiating or promoting tumorigenic processes. Additionally, other metabolites may behave as chemoprotectors, such as the result of 2-hydroxylation in E1 and E2 metabolism [70].

Therefore, the differential expression of CYP1A1 mRNA in control versus treated cells was validated by RT- Real Time PCR (Figure 6A). Next, we investigated whether the changes at the RNA level were translated into protein. PCE treatment for 24 h led to a very modest increase in CYP1A1 protein levels (1.2-fold). A time course incubation during 24, 48, 72 and 96 h led to an increase in CYP1A1 protein in MCF-7 cells of 3.9-fold after 48 h (Figure 6B). The difference between mRNA levels and the corresponding protein levels may indicate that many of the mRNA molecules do not reach

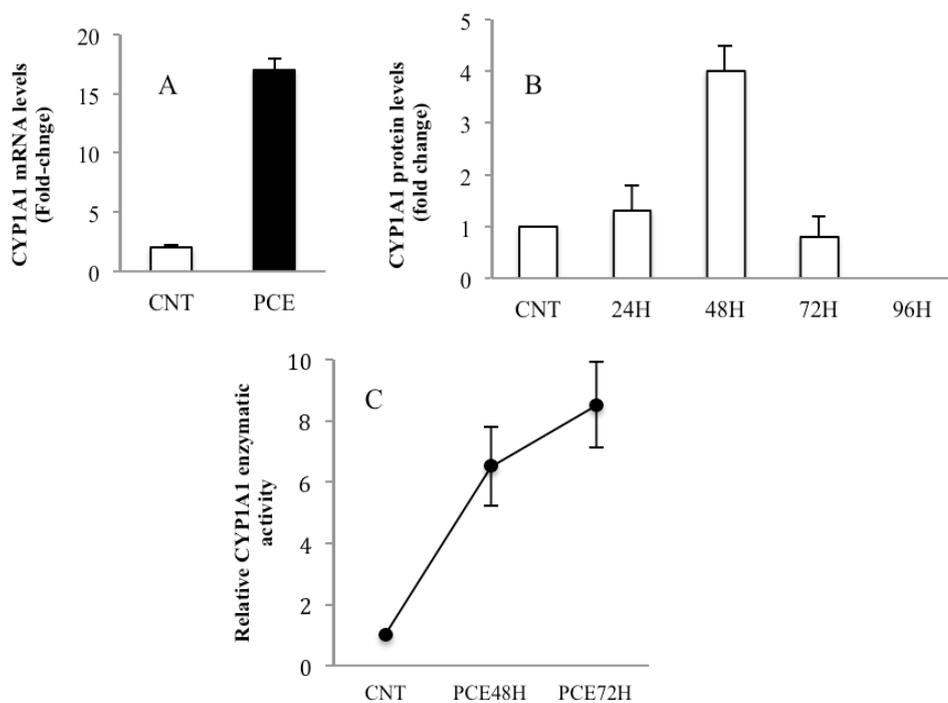


Figure 6. CYP1A1 overexpression in MCF-7 cells treated with PCE. (A) Determination of CYP1A1 mRNA levels. Results are expressed in fold changes compared to MCF-7 control and are the mean \pm SE of 3 different experiments. (B) Determination of CYP1A1 protein levels. Results represent the mean \pm SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA plus post hoc Bonferroni comparison. (C) Determination of CYP1A1 activity in MCF-7 treated cells. Results are expressed relative to the activity of the control and represent the mean \pm SE of 3 different experiments. Significant differences at all time points were evaluated by ANOVA, plus post hoc Bonferroni comparison.

the translational machinery, probably because the translation mechanism is saturated in these conditions. Finally, CYP1A1 activity was determined upon incubation with PCE. An increase in CYP1A1 activity in good correlation with the observed increased in CYP1A1 protein levels was determined for both cell lines (Figure 6C).

The changes in CYP1A1 expression upon incubation with PCE could explain the antioxidant effect of flavonoids at the molecular level since this gene is involved in different oxidative pathways. Additionally, CYP1A1 overexpression might interfere with estrogen metabolism and the production of estrogen metabolites in breast cells. The increase in CYP1A1 activity may shift estrogen metabolism toward the production of 2-OHE2 (2-hydroxyoestradiol), a relatively non-genotoxic metabolite [71].

Finally, we wanted to test whether cocoa polyphenols would exert a synergistic effect in combination with Tamoxifen (TAM) since it has been previously described in breast cancer cells. Thus, MCF-7 cells were incubated with increasing concentrations of TAM (10^{-6} – 10^{-3} M) either alone or in combination with PCE (250 ng/ μ L). Then, cell viability was determined after 48 h. The presence of PCE, which did not cause significant cell death by itself, increased the cytotoxic effect of TAM in MCF-7 cells (Figure 7).

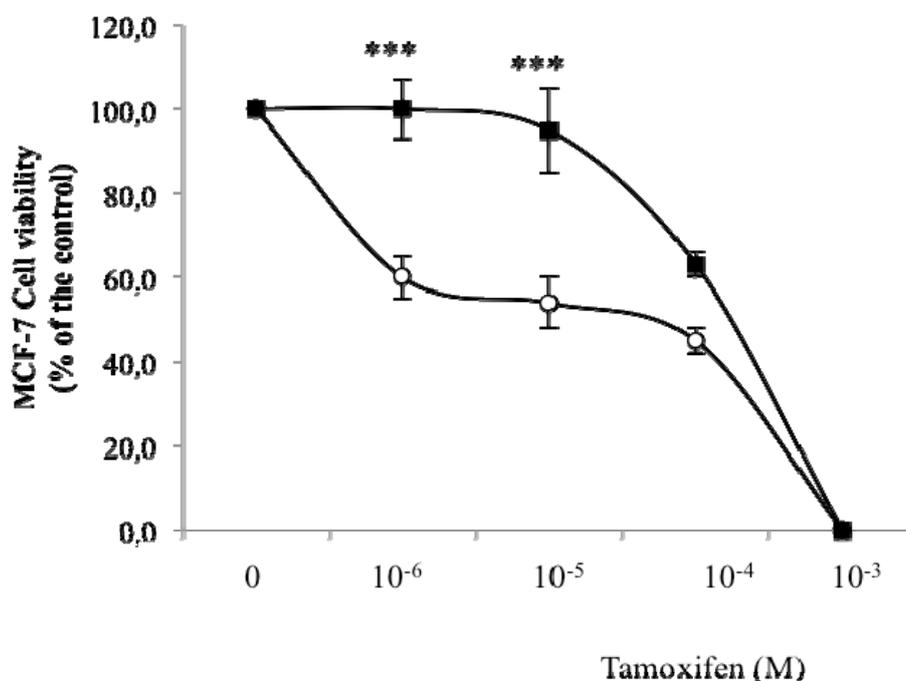


Figure 7. Effect of tamoxifen plus PCE on MCF-7 viability. Tamoxifen (TAM) either alone (filled squares) or in combination with PCE (250 ng/ μ L for 24H, empty circles). Results are expressed as % of living cells compared to the control only with DMSO (0.22%) and represent the mean \pm SE of 3 different experiments. *** p <0.001.

The reduction in cell viability reached an increase of 44% when combined with 10^{-6} M TAM. Thus, in our conditions, the cytotoxic effect of TAM was enhanced by the combination with PCE in MCF-7 cells. The presence of PCE caused a synergistic effect, confirmed by the Chou-Talay method, which led to a decrease in cell viability of up to 40% in MCF-7 cells at tamoxifen concentrations that did not affect cell viability by themselves. A plausible explanation of the synergistic effect observed could be that the increase in estrogen metabolism, induced by the PCE on CYP1A1, could lead to the reduction in the levels of estrogens in mammary tumours, thus contributing to the cytotoxic effect of tamoxifen. Nevertheless, further *in vivo* studies are necessary to analyse the synergism between tamoxifen and cocoa and to establish the possible benefits of cocoa polyphenol consumption during breast cancer therapy.

3. Conclusions

Current global trends in food consumption may have an impact on disease progressions observed worldwide. The impact may occur because of gene regulation caused by nutrients, or by other unclear means that are yet to be discovered. The “omics” and associated technology will surely provide a greater understanding of the environmental and behavioral factors that influence phenotype and its relationship to health and wellness. It is highly likely that during the next decade the nutritional supplement and functional food industries will experience robust growth in response to advances in nutritional genomics research and its applications.

Parallel to this growth will be impressive progress in understanding the specific influence of certain food components on metabolic pathways and their role in health and disease. It will become increasingly less expensive to generate genetic information about individual persons, and such data are likely to redefine the current concept of preventive medicine. Moreover, through nutrigenomic research, new nutritional regulation of gene expression will hopefully come to light. If specific gene regulation by nutrients is identified in genes closely related to disease onset and progression, new arenas for disease prevention and potential for treatment will come to the foreground of nutrition and preventive medicine. Discoveries made in the field of nutrigenomics and nutrigenetics should translate into more effective dietary strategies to improve overall health by identifying unique targets for prevention.

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