

Nutrigenomics to reveal the effects of grape consumption in healthy subjects

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Abstract. The Mediterranean diet places fruit and vegetables as the basis of daily nutrition. Table grape is a typical fruit of the Mediterranean tradition and is consumed worldwide. The CREA Research Centre for Viticulture and Enology has conducted nutrigenomics studies in recent years to investigate the effects of table grapes on human health. This note summarises two trials in which healthy subjects ate the black seedless grape *Autumn Royal* for three consecutive weeks. For our first nutrigenomic study, we used the microarray technique to analyze thousands of genes' expressions simultaneously. The results showed that 463 genes were modulated, and one month after the end of the grape-rich diet, this number almost doubled, reaching 849 genes. Furthermore, more than 200 of these genes are non-coding RNAs important in regulating gene expression. The second nutrigenomic study was conducted to evaluate the effects of grape intake on the expression of microRNAs, identifying 20 circulating microRNAs modulated, most of which were implicated in cancer development. Our results showed that grape intake exerts beneficial effects by modulating genes involved in critical physiological processes such as the immune response, inflammation, autophagy, DNA repair, and mitochondrial functionality.

1 Introduction

Grape (*Vitis vinifera* L.) is a typical fruit of the Mediterranean tradition as well as being consumed all over the world. Since 2000 the world production of table grapes has almost doubled, with China representing the primary world producer with 9.5 million tons and Italy, the leader in European production, in seventh place with 1.1 million tons (OIV data). Fresh table grape and grape-derived products are notably rich in polyphenols, such as flavonoids, phenolic acids, and stilbenes. They are known to benefit human health, mainly attributed to their antioxidant and anti-inflammatory activities [1,2].

The Mediterranean Diet, Intangible Cultural Heritage of Humanity since 2010, represents a healthy dietary pattern mainly characterized by high consumption of vegetables, fruits, cereals, legumes, nuts, and olive oil. Many observational epidemiological studies have shown that the Mediterranean Diet is

associated with a protective effect on chronic diseases such as cancer, cardiovascular disorders, and obesity. However, what are the molecular mechanisms whereby this Diet may exercise its effects is yet to be discovered.

Thanks to the Human Genome Project [3], and the Genome-wide association studies, the sequencing of the entire human genome has enabled the understanding of multiple mutual relations among genes, nutrition, and diseases [4]. These interactions between nutrition and genome have led to two new science branches in nutrition sciences: nutrigenetics and nutrigenomics. Nutrigenetics is defined as the science of the effect of genetic variation on dietary response [5]. Nutrigenomics studies the relationship between genome and Diet and the consequences of nutrients and bioactive food compounds on gene expression, allowing us to decipher how much DNA is influenced by what we eat. It is a science with enormous potential to

prevent or improve health problems starting with nutrition.

Specifically, transcriptomics analyses the effect of a specific compound, food, or Diet on gene expression (upregulation or down-regulation).

Many publications reported altered gene expression after the consumption of polyphenols such as quercetin [6], hesperidin [7], grape seed extract [8], isoflavones [9], olive oil [10,11], resveratrol [12], and mixtures of resveratrol, green tea extract, alpha-tocopherol, vitamin C, n-3 PUFA and lycopene [13].

Recent studies suggest that polyphenols can modulate microRNA (miRNA) profiles. miRNAs are a class of evolutionarily conserved, small non-coding RNAs of 19–24 nucleotides in length that regulate gene expression mainly at the posttranscriptional level. In the cell, they can hybridize with complementary sequences in mRNA and silence genes by destabilizing mRNA or preventing mRNA translation. Several miRNAs have recently been associated with pathological conditions like metabolic syndrome, cardiovascular diseases, and cancer. Moreover, these single-stranded molecules have been found in extracellular human body fluids (e.g., saliva, serum, plasma, and urine) functioning as chemical messengers to mediate cell-cell communication [14]. They are known to control different processes such as cell cycling, programmed cell death, cell differentiation, tumour development, metastasis, and sensing of nutrient stress [15], so they are considered crucial molecular elements of human physiology and disease.

Surprisingly, only a few studies have investigated the effects of table grape intake on human health. The CREA Research Centre for Viticulture and Enology has conducted two human studies in recent years in collaboration with hospital structures to investigate the effects of table grape consumption on health. The aim was to identify the molecular mechanisms modulated by grape polyphenols using a nutrigenomics approach.

In this note, we summarise two separate trials in which healthy subjects consumed the black seedless grape Autumn royal for three consecutive weeks, limiting the intake of other polyphenols. The first work evaluated changes in the gene expression profile of the total transcriptome [16], and the second investigated the influence of table grape intake on the circulating miRNA profile [17].

2 Materials and methods

2.1 Table grape

The grapes used in the two trials were produced in the Apulia region in the province of Bari in compliance with the regional regulations for integrated table grapes production. *Autumn Royal* was chosen for its high polyphenolic content and high antioxidant properties. Fresh grape was distributed weekly to the participants during harvest time (September 2014, September 2019). The volunteers were instructed to keep grape servings in their home refrigerator at 4°C until consumption.

2.2 Study design

Both nutrigenomic studies enrolled healthy volunteers invited to daily consumption of 5 g of fresh table grapes per kg of body weight for 21 days. Blood from the subjects was collected at baseline (T₀), after 21 days of a grape-rich diet (T₁), and after a one-month washout (T₂) (Fig. 1). The ethical committee approved the study protocols, and written informed consent was obtained from participants before starting the study.



Figure 1. Pilot study design.

The first study, a "pilot study," recruited 20 healthy subjects and started in September 2014 and finished in November 2014.

The second nutrigenomic study was a randomized controlled trial in which 40 consecutive subjects were recruited voluntarily and randomly assigned to two study groups. The control group received only dietary recommendations, and the grape group received a daily dose of fresh table grapes. The studies started in September 2019 and finished in November 2019.

2.3 Microarray analysis

In the 2014 study, using a transcriptomic approach, we evaluated the changes in gene expression of peripheral blood mononuclear cells (PBMCs) from six subjects (three males and three females) at baseline (T₀), after three weeks of grape-rich diet (T₁), and after one-month washout (T₂). Total RNA was isolated from PBMCs using the PureLink® RNA Mini Kit, according to the manufacturer's instructions (Ambion by Life Technologies, Carlsbad, CA, USA). For microarray experiments, we applied the Two-Color Microarray-Based Gene Expression Analysis (Version 6.7) protocol (Agilent Technologies, Santa Clara, CA, USA). The Gene Group Functional Profiling (g:GOST) and the Compact Compare of Annotations (g:Cocoa) tools available in the g:Profiler web server were used for the bioinformatic functional analyses. Quantitative real-time PCR (qRT-PCR) was performed to validate microarray analysis. All reactions were performed on the stepOne Plus PCR Real Time (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol using Brilliant III Ultra-fast SYBR® Green qPCR Master Mix (Agilent, Santa Clara, CA, USA) and gene-specific primers.

2.4 Analysis of circulating miRNAs

In the 2019 study, circulating miRNAs levels are detected by Real-Time quantitative PCR (RT-qPCR) (Qiagen, Hilden, Germany). Total RNA was isolated from serum of volunteers using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden,

Germany). The bioinformatic functional analysis was carried out with the miRNA Pathway Dictionary Database (miRPathDB). All analyses are performed at baseline and after 21 days of dietary treatment.

3 Results and Discussion

3.1 Gene expression study

In the pilot study, gene expression profiling of peripheral blood mononuclear cells (PBMCs) identified 930 differentially expressed transcripts. Microarray experiments showed that after 21 days of a fresh table grape-rich diet, 463 genes are modulated while, one month after the end of the grape-rich diet, this number almost doubled, reaching 849 genes (Table 1) [16,18]. The changes in gene expression more pronounced after the washout period could be explained as a consequence of an indirect effect of grape, e.g. through the modulation of regulatory genes, which activate other genes. This long-lasting effect of dietary supplementation is supported by previous findings by our own group, on the same subjects [19], and others [19]. Among modulated genes, more than 200 are long non-coding RNAs (lncRNAs), almost all downregulated after the washout period when polyphenols' direct effect is supposed to be completely exhausted [21].

Table 1. Number of genes differentially expressed at different timeframes.

Timeframe	up-regulated		down-regulated		total genes
	protein coding genes	long non-coding RNAs	protein coding genes	long non-coding RNAs	
T1vsT0	178	24	186	75	463
T2vsT0	362	27	317	143	849

The functional analysis of differentially expressed genes revealed significant changes in processes critical for organismal and cell well-being, such as inflammation and, immunity, thrombosis. Firstly, the results supported the antithrombotic and anti-inflammatory activity reported by Ammollo et al. Concerning the anti-inflammatory activity of the grape-rich diet, both array analysis and qRT-PCR obtained showed a downregulation of the IL-1 β gene and a significant enrichment in GO:BPs related to leukocyte cell adhesion and cytokine production. These findings suggest that table grape intake could mitigate the low-grade generation of proinflammatory signals induced in healthy subjects by many subtle stimuli coming from daily life and which contribute to processes like atherosclerosis and ageing. Furthermore, microarray analysis revealed additional protective mechanisms induced by table grape intake beyond mitigation of inflammation and thrombosis, such as autophagy, DNA repair and mitochondrial biogenesis.

Long non-coding RNAs have a broad spectrum of regulatory functions. Our data revealed the presence of 75 lncRNAs after grape intake and 143 at the end of the washout period. The most significant number of downregulated lncRNAs were found at the end of the washout period. The analysis of lncRNAs downregulated in our study revealed that many of them are over-expressed in many types of tumours, and chronic diseases due to the persistency of inflammation and metabolic syndromes such as obesity. Altogether, these findings provide exciting clues for the crucial role of ncRNAs in grape intake's long-term effects on a series of biological processes.

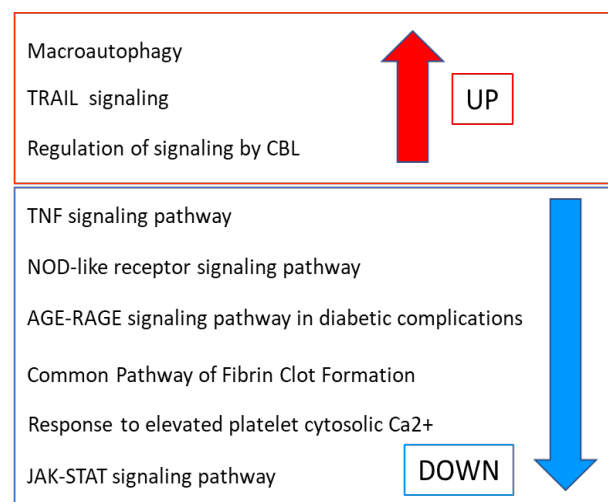


Figure 2. Most relevant pathways of differentially expressed genes as revealed by functional analysis.

These results were of great interest and made it possible to state that the intake of table grapes induces benefits for human health in the short and, above all, in the medium and long term. Nutrigenomics confirmed the biochemical data on coagulation processes previously obtained on the same subjects about the antithrombotic and anti-inflammatory effects of grape intake [19].

4 microRNAs expression study

The second nutrigenomic study evaluated the effects of fresh table grape consumption for three weeks in healthy subjects on circulating levels of the most common miRNAs. We also explored the miRNAs modulated function by in silico analysis to discover the regulatory network governed by these miRNAs [17].

All analyses are performed at baseline and after 21 days of dietary treatment. Circulating miRNAs levels are detected by Real-Time quantitative PCR (RT-qPCR) followed by bioinformatic functional analysis.

The study identifies 20 circulating miRNAs differentially expressed in healthy subjects after grape intake, and in particular, 18 of 20 are down-regulated and 2 are up-regulated (Fig. 3).

miR-208a-3p miR-33a-5p	UP
miR-181a-5p miR-30e-5p miR-30d-5p miR-335-5p miR-222-3p miR-15a-5p miR-421 miR-339-5p miR-378a-3p let-7f-5p miR-29b-3p miR-106b-3p miR-324-5p miR-1260a miR-365a-3p miR-155-5p miR-335-3p miR-200c-3p	DOWN

Figure 3. microRNAs significantly modulated (p -value < 0.05) in the grape group.

To obtain a rapid overview of the enriched pathways significantly modulated by our specific miRNAs set, we performed a Bioinformatics Functional Analysis using mirPathDB and Kyoto Encyclopedia of Genes and Genomes (KEGG) database, showing that the miRNAs involved in the regulation of a significant number of pathways were miR-29b-3p, miR-181a-5p, miR-365a-3p, miR-378a-3p, miR-15a-5p; the pathways modulated by the major number of miRNAs were “microRNA in cancer” and “pathways in cancer.” The network of interactions between all miRNAs and target genes showed numerous connections among the analyzed miRNAs. Our study showed that the three-week grape-rich Diet modulated several miRNAs synergistically, triggering many cellular mechanisms.

Finally, to better understand the effects of grape intake on circulating miRNAs levels, we performed Venn diagrams considering target genes (Fig. 4A) and enriched pathways (Fig. 4B) of only five miRNAs involved in modulating the most significant number of pathways. It is interesting to note that all the five miRNAs regulated 12 significant pathways out of 89, for example, “Focal adhesion,” “PI3K-Akt signaling pathway,” “ForkheadBox O (FoxO) signaling pathway,” and numerous others primarily involved in cancer such as “microRNA in cancer,” “pathways in cancer,” “colorectal cancer” and “pancreatic cancer”.

Overall, these results suggested that the grape-rich Diet, by the down-regulation of several miRNAs that possess pleiotropic and synergistic activity, could greatly impact human health, triggering mechanisms such as anti-inflammatory, pro-autophagic, anti-proliferative, and anti-atherosclerosis activities.

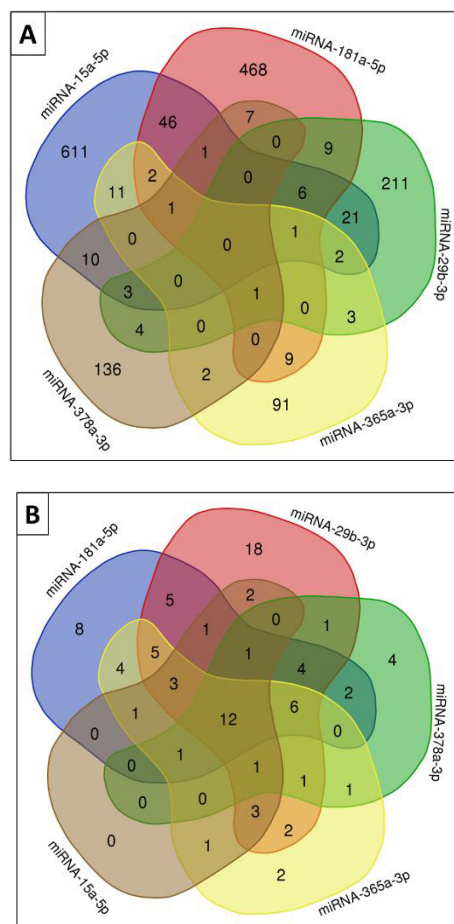


Figure 4. Venn diagrams of target genes (A) and significant pathways (B) of miR-29b-3p, miR-181a-5p, miR-365a-3p, miR-378a-3p, and miR-15a-5p.

5 Conclusion

Investigating the role of nutrition and Diet in the modulation of genes and miRNA profiles remains a relatively new field of research. Furthermore, exploring the link between natural dietary compounds and transcriptome is mandatory. Our studies support the concept that table grape intake induces health benefits. More importantly, the transcriptomic findings suggest that the mechanisms underlying these beneficial effects may involve numerous strategic processes such as immune response, autophagy, DNA repair and mitochondrial functionality.

A grape-rich diet may exert its beneficial effects also by modulating the levels of circulating miRNAs targeting different strategic metabolic pathways, particularly the miRNAs related to pathways involved in counteracting cancer development, including gastrointestinal cancers. Furthermore, the analysis of circulating miRNAs offers promising evidence that paves the way for future studies on the close relationship between dietary table grapes and human disease prevention. Circulating miRNAs may serve as disease biomarkers and play important roles in intercellular communication.

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