MicroRNAs' Gene Expression Technology for the Evaluation of Disease Modulation by Mild Nutrients

Farid E Ahmed¹, Mostafa M Gouda^{2,3}, Laila Hussein², Paul W Vos⁴, Mohamed Mahmoud⁵, and

Nancy C Ahmed¹

¹GEM Tox Labs, Institute for Research in Biotechnology, 2905 South Memorial Drive, Greenville, NC 27834, USA.

²Department of Nutrition & Food Science, National Research Center, El-Bohouth Street, Dokki, Cairo, Egypt

³National Research and Development Center for Egg Processing, College of Food Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, PR, China

⁴Department of Biostatistics, College of Allied Health Sciences, East Carolina University, 600 Moye Boulevard, Greenville, NC 27858, USA

⁵USDA/ARS Children's Nutrition Research, 1100 Bates Street, Houston, TX 77030, USA

Received: June 19, 2018; Accepted: July 12, 2018; Published: July 21, 2018

***Corresponding author:** Farid E Ahmed, GEM Tox Labs, Institute for Research in Biotechnology, 2905 South Memorial Dr, Greenville, NC 27834, USA, Tel. 252-375-9656, E-mail: gemtoxconsultants@yahoo.com

Abstract

This article demonstrates the use of melt curve analysis (MCA) for the interpretation of mild nutrigenomic micro (mi) RNA gene expression data, by measuring the magnitude of the expression of key miRNA molecules in stool of healthy human adults, used as molecular markers in excrements, following the intake of polyphenol-rich Pomegranate juice (PGJ), functional fermented sobya (FS), which is rich in potential Probiotic lactobacilli, or their combination. Total small RNA was isolated from stool of 25 volunteers before and following a three week dietary intervention trial. Expression of 88 miRNA genes was evaluated using Qiagen's 96 well plate RT2 miRNA qPCR arrays. Employing parallel coordinates plots, no significant separation for the gene expression (C_q) values was observed using Roche 480@ PCR Light Cyclers instrument used in this study, and none of the miRNAs showed significant statistical expression after controlling for the false discovery rate. On the other hand, melting temperature profiles produced during PCR amplification run, found seven significant genes (miR-184, miR-203, miR-373, miR-124, miR-96, miR-379 and miR-301a), which separated candidate miRNAs that could function as novel molecular markers of relevance to oxidative stress and immunoglobulin function, for the intake of polyphenol (PP)-rich, functional fermented foods rich in lactobacilli (FS), or their combination. We elaborate on these results, and present a detailed review on use of melt curves for analyzing nutrigenomic miRNA expression data, which initially appear to show no significant expressions, but are actually more subtle than this simplistic view, necessitating the appreciation of the important role of MCA for a comprehensive understanding of what the collective expression and MCA data collectively imply. We have correlated miRNA genes with messenger (m) RNA genes using bioinformatics' methods.

Key Words: biomarkers, DNA, melt curve, fermented sobya, miRNA, pomegranate, PCR, RNA

Introduction

Gene expression and its control by miRNAs

Cell's gene expression profile determines its function, phenotype and cells' response to external stimuli, and thus helps elucidate various cellular functions, biochemical pathways and regulatory mechanisms [1]. Several gene expression profiling methods at the mRNA level have emerged during past years, and have been successfully applied to cancer research. Profiling by microarrays [2, 3] allows for the parallel quantification of thousands of genes from multiple samples simultaneously, using a single RNA preparation, and has become valuable because microarrays are convenient to use, do not require large-scale DNA sequencing, gives a clear idea of cells' physiological state, and is considered a comprehensive approach to characterize cancer molecularly, as seen in studies on colon cancer [1-3].

Control of gene expression has been studied by miRNA molecules, a small non-coding RNA molecules (18-24 nt long), involved in transcriptional and post-transcriptional regulation of gene expression by inhibiting gene translation, and the discovery of this molecule resulted in a 2006 Noble Prize in Physiology & Medicine to Andrew Z Fire and Craig C. Mello for their work on RNA interference (RNAi) [https://science.howstuffworks.com/environmental/conservation/issues/nobel-prize-rnai.htm]. MiRNAs silence gene expression through inhibiting mRNA translation to protein, or by enhancing the degradation of mRNA. Since first reported in 1993 [4], the number of identified miRNAs in June 2014, version 14.0, the latest miRBase release (v20) [5] contains 24,521 miRNA loci from 206 species, processed

to produce 30,424 mature miRNA products. MiRNAs are processed by RNA polymerase II to form a precursor step which is a long primary transcript. Pri-miR is converted to miRNA by sequential cutting with two enzymes belonging to a class of RNA III endonuclease, Drosha and Dicer. Drosha converts the long primary transcripts to ~70 nt long primary miRNAs (pri-miR), which migrate to the cytoplasm by Exportin 5, and converted to mature miRNA (~22 nt) by Dicer [6]. Each miRNA may control multiple genes, and one or more miRNAs regulate a large proportion of human protein-coding genes, whereas each single gene may be regulated by multiple miRNAs [7]. MiRNAs inhibit gene expression through interaction with 3'-untranslated regions (3' UTRs) of target mRNAs carrying complementary sequences [7]. Thus, the tumors had figured out a shrewd way to turn on the miRNAs, creating a growth process that is impossible to stop.

Effect of antioxidant polyphenols --abundant in Mediterranean diets-- on gene expression unraveled by the availability of molecular biology techniques, reveals our adaptation to environmental changes [8]. Efforts to study the human transcriptome have collectively been applied to tissue, blood, and urine (i.e., normally sterile materials), as well as stool (a non-sterile medium). Extraction protocols that employ commercial reagents to obtain high-yield, reverse-transcribable (RT) RNA from human stool in studies performed on colon cancer have been reported [1, 2, 9]

Micro (mi) RNAs as biomarkers, and their roles in disease processes

A biomarker is believed to be a characteristic indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic interventions [10, 11]. In contrast, clinical endpoints are considered as variables representing a study subject's health from his/her perspective [12-22]. A variety of biomarkers exist today as surrogates to access clinical outcomes in diseases, predict the health of individuals, or improve drug development. An ideal biomarker should be safe and easily measured, is cost effective to follow up, is modifiable with treatment, and is consistent across genders and various ethnic groups. Because we never have a complete understanding of all processes affecting individual's health, biomarkers need to be constantly reevaluated for their relationship between surrogate endpoints and true clinical endpoints [12-14]. MiRNAs have been used herein as biomarkers for assessing the effect of intake of PP-rich or fermented foods on the expression of 88 miRNA genes known to influence cancer.

Disease modulation by nutrients

Cardiovascular diseases due to hypercholesterolemia are considered a risk factor for Chronic Heart Disease (CHD), and chronic degenerative diseases --caused wholly or partially by dietary patterns-- represent the most serious threat to public health [23]. Moreover, nearly one-third of all cancer deaths are due to poor nutrition, lack of physical activity, and obesity; and these risk factors account for nearly 80% of large intestine, breast, and prostate cancers [24]. Chronic inflammation is

considered a common factor that contributes to the development and progression of these illnesses, which are caused by and/or modified by diet [25].

Pomegranate juice (PGJ) and derived products are considered the richest sources of polyphenolic compounds [26], with positive implication on total serum cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) plasma lipid profile [27]. Moreover, anthocyanin and ellagitannins pigments, mainly punicalagins, inhibit the activities of enzymes 3-hydroxy-3-methylglutaryl-CoA reductase and sterol O-acyltransferase, important in cholesterol metabolism [28]. Probiotic bacteria also contribute to lowering plasma hyper cholestolemia due to the above mechanism, caused by the Probiotic bile salt hydrolases (BSH) activity. This Probiotic enzyme hydrolyses conjugates both glycodeoxycholic and taurodeoxycholic acids to hydrolysis products, inhibiting cholesterol absorption and decreasing reabsorption of bile acid [29].

Colonic micro biota is a central site for the metabolism of dietary PP and colonization of Probiotic bacteria. A dietary intervention study with Probiotic strains from three *Lactobacillus* species (*L. acidophilus*, *L. casei* and *L. rhamnosus*) given to healthy adults, showed that bacterial consumption caused the differential expression of from hundreds to thousands of genes in vivo in the human mucosa. The interaction of PP with the gut micro biota influences the expression of some human genes (i.e., nutritional transcriptomics), which mediates mechanisms underlying their beneficial effects [30]. Similar in vivo mucosal transcriptome findings have been reported when adults were given the Probiotic *L. plantarum*, illustrating how probiotics modulate human cellular pathways, and show remarkable similarity to responses obtained for certain bioactive molecules and drugs [31].

Materials and Methods

Participants

Study subjects were 25 healthy adults, 20 to 34 years old; exclusion was: absence of metabolic diseases, no use of medication for the last 6 weeks, and no signs of allergy or hypersensitivity to food or ingested material.

Exclusion criteria at the time of the screening were as follows: History of diabetes, hypertension, heart disease, or endocrine disorders; abnormal blood chemistry profile, fasting LDL-cholesterol concentration > 3.37 mmol/L (> 130 mg /d L), or fasting triacylglycerol concentration > 3.39 mmol/L (> 300 mg/dL), taking antioxidant or fish oil supplements. Female subjects were neither pregnant nor lactating. To minimize the potential confounding effects of consuming fluctuating amounts of foods and beverages that are high in dietary flavonoids, all subjects avoided the intakes of purple grapes, cocoa and chocolate during the entire three week dietary intervention trial. The volunteers were instructed to continue to eat their normal diet and not to alter their usual dietary or fluid intake with the exception of the previously mentioned food restrictions.

Compliance with the supplementation in all subjects was satisfactory, as assessed daily, and all subjects continued their habitual diets throughout the study. The research protocol was approved by the institution review board at Egypt's NRC, and all subjects have given written consent prior to their participation in the study.

Design of the study

Figure 1 shows the design of the nutrigenomic randomized

study. Estimated dietary intake was assessed by 3 repeated food records, one week before they were enrolled in the trial. The average portion sizes consumed, as well as composition data values from nutrient composition of the food were combined to assess average daily energy and nutrient intakes by the "nutrisurvey" software program. The characteristics of the voluntary subjects who were enrolled in the study, the mean daily energy intake, as well as selected macro nutrients are presented in Table 1.

Isolate RNA→Reverse transcribe total RNA into cDNA→ Carry out NGS or microarray studies →Analyze NGS or microarray data→ Chose a smaller number of samples to run RT-qPCR reactions→Analyze quantitative PCR data

Figure 1: miRNA Quantitation Experimental Workflow

Table 1: Composition of the supplements

Parameter	Unit	Dietary supplements			
		Control (portion served)	FS (portion served)	PGJ (portion served)	FS+ PG (portion served)
Portion Size	g	-	170	250	150+10
Total Solids	g	-	40.01	17.75	48
Carbohydrate	g	-	51.10	32.75	59
Dietary Fiber	g	-	54	0.25	48.6
Energy	kcal	-	263	135	290
Lactobacillus	cfu/	-			
diverse	serving size	-	5.1 x 10 ⁹	-	4.5 x10 ⁹
Yeast	cfu/serving size	-	2.77 x10 ¹⁰	-	2.44 x10 ¹⁰
Total PP	mg*/portion g	-	-	519.1±8.75	207.65±3.5
Antioxidant		-			
activity	(AEAC)**	-	7.74±1.33	11.35±2.2	11.37±2.2

Supplements

Pomegranate was obtained in bulk from the Obour Public Market, Cairo, Egypt. Pomegranate fruits were peeled and the juice was extracted using a laboratory pilot press (Braun, Germany). The juice was distributed in aliquots of 100 or 250 grams in air tight, light-proof polyethylene bottles, and frozen at -20°C, where pomegranate polyphenols remained stable. Soursobya, a fermented rice porridge containing per gram 3×10^7 cfu diverse lactic acid bacteria (LAB) and 1×10^7 cfu *Sacharomyces cerevisiae*. With added ingredients such as milk, sugar and grated coconut, was purchased twice a week from the retail market, and saved in the refrigerator. Sobyia is fermented rice. Table 2 illustrates the proximate initial and final mean urinary polyphenols, plasma and urinary ant oxidative activity, urinary thiobarbituric acid reactive species (TBARS), and erythrocytic glutathione-S-transferase (GST).

Urine, blood and stool collection & storage

One day before starting the trial, urine and stool samples were collected from all volunteers, processed according to our standard operating methods, and saved frozen at -70°C.

Urine samples: Collection begins in the early morning after the subjects had fasted 10–12 h both on -1, and +21 d nutritional intervention and aliquots (2 mL) were immediately frozen at -20°C for biochemical analysis.

Blood samples: Blood was drawn by vein-puncture and collected in sodium citrated tubes. The plasma was separated from blood cells by centrifugation at 3000 rpm for 15 min at 4°C and the separated plasma was stored at -70°C for later biochemical analysis. The red blood cells (RBCs) were washed using cold physiological saline solution and stored at -20°C.

Table 2: Initial and final mean urinary polyphenols, plasma and urinary ant oxidative activity, urinary TBARS and erythrocytic GST

Parameter	Unit	Control		Sobya		Pomegranate		Sobya+Pomegranate	
		Baseline Final		Baseline Final		Baseline Final		Baseline Final	
		X±SE	X±SE p	X±SE	X±SE p	X±SE	X±SE p	X±SE	X±SE p
Urinary	GAE/mg	10.36	8.11	11.84	9.86	5.70	55.23 <0.05	10.40	21.62
polyphenol	creat	±1.8	±2.2	±6.2	±1.8	±1.4	±21.7	±3.2	±7.3
Urinary	AEAC/mg	9.74	8.13	3.89	10.30	7.18	46.57 <0.05	10.90	20.25
antioxidant activity	creat*	±2.0	±2.7	±0.9	±2.3	±0.9	±18.0	±2.4	±3.9
Urinary	ug/mg	83.04	75.17	82.77	29.97	173.93	51.48 <0.05	157.70	40.62
TBARS	creat	±12.1	±15.3	±27.8	±4.4	±44.8	±8.2	±47.8	±8.3
Plasma	AEAC/	6.36	5.99	3.70	4.55	3.64	5.92 <0.05	2.78	4.49
antioxidant	100 ml	±2.81	±2.66	±0.33	±0.27	±0.30	±0.68	±0.11	±0.58
E-GST	IU/g Hb	5.94	5.45	4.26 <0.05	7.21	4.73	8.34	4.56	6.90
activity		±3.3	±4.1	±0.5	±0.8	±1.0	±1.0	±1.0	±1.0

X±SE: Mean ± Standard Error, *:mmol ascorbic acid equivalent antioxidant capacity/mg creatinine; GAE: gallic acid equivalent, Mean values are significantly different if the P-values are less than 0,05 (P<0.05) [13].

Stool samples: Feces (excrement) were obtained from the 25 healthy adults, twice at day 0 and three weeks after the dietary intervention. All stools were collected with sterile, disposable wood spatulas in clean containers, after stools were freshly passed, and then placed for storage into Nalgene screw top vials (Thermo Fisher Scientific, Inc., Palo Alto, CA, USA), each containing 2 ml of the preservative RNA later (Applied Biosystems/Ambion, Austin, TX, USA), which prevents the fragmentation of the fragile mRNA molecule [1], and vials were stored at - 70 °C until samples were ready for further analysis. Total small RNA, containing miRNAs, was extracted from all frozen samples at once, when ready, and there was no need to separate mRNA containing small miRNAs from total RNA, as small total RNA was suitable daytracking small total RNA from stool was carried out using a guanidinium-based buffer, whand saved frozen atich comes with the RNeasy isolation Kit®, Qiagen, Valencia, CA, USA, as we have previously detailed. DNase digestion was not carried out, as our earlier work demonstrated no difference in RNA yield or effect on RT-PCR after DNase digestion [1]. The time to purify aqueous RNA from all of the 25 frozen stool samples was ~ three hours. Small RNA concentrations were measured spectrophotometrically at λ 260 nm, 280 nm and 230nm, using a Nano-Drop spectrophotometer (Themo-Fischer Scientific). The integrity of total RNA was determined by an Agilent 2100 Bio analyzer (Agilent Technologies, Inc., Palo Alto, CA, USA) utilizing

the RNA 6000 Nano Lab Chip®. RNA integrity number (RIN) was computed for each sample using instrument's software [1].

Preparation of ss-cDNA for molecular analysis

The RT² miRNA First Strand Kit® from SABiosciences Corporation (Frederick, MD, USA) was employed for making a copy of ss-DNA in a 10.0 µl reverse transcription (RT) reaction, for each RNA samples in a sterile PCR tube, containing 100 ng total RNA, 1.0 µl miRNA RT primer & ERC mix, 2.0 /µl 5X miRNA RT buffer, 1.0 µl miRNA RT enzyme mix, 1.0 µl nucleotide mix and Rnase-free H₂O to a final volume of 10.0 µl. The same amount of total RNA was used for each sample. Contents were gently mixed with a pipettor, followed by brief centrifugation. All tubes were then incubated for 2 hours at 37°C, followed by heating at 95°C for 5 minutes to degrade the RNA and inactivate the RT. All tubes were chilled on ice for 5 minutes, and 90 µl of Rnase-free H₂O was added to each tube. Finished miRNA First Strand C DNA synthesis reactions were then stored overnight at -20°C [1].

Use of cancer RT² miRNA PCR array 96-well plate to study miRNAs' expressions

We used a SABiosciences RT² miRNA qPCR Array Plate System for Human (Qiagen) to analyze miRNA expression using real-time, reverse transcription PCR (RT-qPCR) as a sensitive and reliable quantitative method for miRNA expression analysis.

The arrays employ a SYBR Green real-time PCR detection system, which has been optimized to analyze the expression of many mature miRNAs simultaneously. Each 96-well array plate contains a panel of primer sets for 88 relevant miRNA focused pathways (one universal primer and one gene-specific primer for each miRNA sequence), plus four housekeeping genes (Human SNORD 48, 47 and 44, and U6), and two RNA and two PCR quality controls. Duplicate RT Controls (RTC) to test the efficiency of the miRNA RT reaction, with a primer set that detects the template synthesized from the built in miRNA External RNA Control (ERC).

There are duplicate RT controls (RTC) to test the efficiency of the miRNA RT process, with a primer set to detect the template synthesized from the kit's built-in miRNA External RNA Control (ERC). There is also duplicate positive PCR controls (PPC) to test the efficiency of the PCR process, using a per-dispensed artificial DNA sequence and the primer set that detects it. The two sets of duplicate control wells (RTC and PPC) also test for inter-well, intra-plate consistency. The human RT² miRNA PCR Arrays reflect miRNA sequences annotated by the Sanger miRBase Release 14. Figure 2 shows the layout of the MAH-102F array.

Layout for Human cancer RT² miRNA qPCR Array (MAH-102A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	let-7a	miR-133b	miR-122	miR-20b	miR-335	miR-196a	miR-125a-5p	miR-142-5p	miR-96	miR-222	miR-148b	miR-92a
B	miR-184	miR-214	miR-15a	miR-18b	miR-378	let-7b	miR-205	miR-181a	miR-130a	miR-199a-3p	miR-140-5p	miR-20a
C	miR-146b-5p	miR-132	miR-193b	miR-183	miR-34c-5p	miR-30c	miR-148a	miR-134	let-7g	miR-138	miR-373	let-7c
D	let-7e	miR-218	miR-29b	miR-146a	miR-212	miR-135b	miR-206	miR-124	miR-21	miR-181d	miR-301a	miR-200c
E	miR-100	miR-10b	miR-155	miR-1	miR-363	miR-150	let-7f	miR-27b	miR-7	miR-127-5p	miR-29a	miR-191
F	let-7d	miR-9	let-7f	miR-10a	miR-181b	miR-15b	miR-16	miR-210	miR-106a/17	miR-98	miR-34a	miR-25
G	miR-144	miR-128a	miR-143	miR-215	miR-19a	miR-193a-5p	miR-18a	miR-125b	miR-126	miR-27a	miR-372	miR-149
H	miR-23b	miR-203	miR-32	miR-181c	SNORD48	SNORD47	SNORD44	RNU6-2	miRTC	miRTC	PPC	PPC

Source: SABiosciences, a Qiagen Company, Fredrick, Maryland, USA

Figure 2: Array lay out for Qiagen's human cancer RT² miRNA qPCR Plate Array (MAH-102A).

Performing real-time quantitative polymerase chain reaction (qPCR)

We used RT² SYBR Green qPCR Master Mix (SBA Biosciences) to obtain accurate results from our qPCR arrays. The following components were mixed in a 15-ml tube for 96-well plate format: 1275 µl of 2X RT² SYBR Green PCR Master Mix, 100 µl of diluted first strand reaction, 1175 µl of ddH₂O (total volume 2550 µl, of which 2400 was needed for 96 reactions, each well having 25 µl, with 150 µl cocktail remaining).

We employed a Roche Light Cycler 480® 96-well block PCR Machine (Roche, Mannheim, Germany) to carry out quantitative real-time miRNA expressions. When ready, we removed the needed miRNA qPCR Arrays, each wrapped in aluminum foil, from their sealed bags, added 25 µl of the same cocktail to each well, adjusted the ramp rate to 1°C/sec. We used 45 cycles in the program, and employed the Second Derivative Maximum method, available with the Light Cycler 480® software for data analysis [32]. We first heated the 96 well plate for 10 min at 95°C to activate the Hot Start DNA polymerase, then used a three-step cycling program (a 15 seconds heating at 95°C to separate the ds DNA, a 30 seconds annealing step at 60°C to detect and record SYBR Green fluorescence at each well during each cycle, and a final heating step for 30 seconds at 72°C). Each plate was visually inspected after the run for signs of evaporation from the wells. Data were analyzed using the 2-ΔΔCt method [33]. Resulting threshold cycle values for all wells were exported to a blank

Excel sheet for analysis. We also ran a Dissociation (Melt) Curve Program after the cycling program [34], and generated a first derivative dissociation curve for each well in the plate, using the LC (Light cycler's®) software.

Use of transcriptomic markers for colon cancer screening in stool, blood and tissue

Using molecular methods to carry out quantitative gene expression studies on stool, blood and tissue samples, allows for sensitive and specific routine monitoring and diagnostic screening of colon cancer at the early malignant tags for ten genes: HPRT, IGF2, FLNA, TGFβ₁, CKS2, CSE1L, KLK10, CXCL3, GUCA2B, IL-12] in a two-step quantitative PCR end-point, on an Applied Bio system 9600 thermo cycler (Foster City, CA) on single-stranded (s s)-cDNA at >90% sensitivity and >95% specificity, using a master mix containing final concentrations of 1X Hugh fidelity PCR buffer, 0.2 mM dNTP, 2 mM MgSO₄, 0.4 µM forward and reverse primers, 0.1 ng ss-cDNA template and 1 U of "hot start" Platinum High Taq DNA polymerase (Invitrogen) in a final volume of 25 µl, in a 100 µl PCR tube. Running conditions were: one cycle at 94°C for 3 min to activate the hot start Taq, 35 cycles of 94°C denaturation for 45 sec, 55°C annealing for 1 min and 72°C elongation for 1 min, followed by one elongation/extension cycle at 72°C for 7 min. Reactions were placed in a well of a 1% agarose gel immersed with 1X Tris-acetate EDTA (TAE) gel running buffer in an electrophoresis apparatus (5 V per cm), gel stained with ethidium bromide (0.25 µg/ml final

concentration), and visualized using an Alpha Innotech charge-coupled device (CCD) based imaging system (San Leandro, CA). A semi-quantitative real-time PCR, which employed the comparative cross point (CP) method, also named E-method [36] was employed on a Roche's Light Cycler (LCTM) model 2.0 PCR using the LC Relative Quantification Software TM; which employs the LC Relative Quantification Software using standard curves in which standard concentrations are plotted versus the threshold cycle to calculate the unknown sample, without user input [36-38], Figure 2 [1]. However, we later on employed the more stable miRNAs' genes, because miRNA molecules are more stable, and easier to handle and work with than the more fragile mRNA molecules. We have bioinformatically correlated the mRNA with miRNA genes using Target Scan algorithm, and employing the DAVID program [39].

Role of biomarker miRNAs in various diseases

MiRNA functions were shown to regulate development [35] and apoptosis [36], and dysregulation of miRNAs has been associated with many diseases such as various cancers [37], heart diseases [38], and kidney diseases [39]. Nervous system diseases [40], alcoholism [41], obesity [42], auditory diseases [43], eye diseases [44], skeletal growth defects [45], as well as key role in host-virus pathogenesis of viral diseases [46]. A negative correlation was found between tissue specificity of interactions and miRNA in a number of diseases, and an association between miRNA conservation and disease, and predefined miRNA groups allow for identification of novel disease biomarkers at the miRNA level [47]. Specific miRNAs are crucial in oncogenesis [48], effective in classifying solid [49] and liquid tumors [50], and function as oncogenes or tumor suppressor genes [51]. MiRNA genes are often located at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common breakpoints regions, suggesting their involvement in carcinogenesis [52]. MiRNAs have shown to serve as biomarkers for cancer diagnosis, prognosis and/or response to therapy [53, 54]. Profiles of miRNA expression differ between normal tissues and tumor types, and evidence suggests that miRNA expression profiles can cluster similar tumor types together more accurately than expression profiles of protein-coding messenger (m) RNA genes [55]. Besides, small miRNAs (~18-22 nt long) are stable molecules than the fragile mRNA [56].

Melt Curve analysis

MCA has been an effective and economical way for identification of virus stains [57], genes [58], bacterial strains [59], insect species [60], temperature validation of PCR cyclers [61], detection of translocations in lymphomas [62] and RNA interference/gene silencing [63]. Thus, the presence of double peaks during MCA is not always indicative of non-specific amplification, and other methods such as agarose gel electrophoresis and use of melt curve prediction software [64, 65] are also needed in order to determine the purity of an amplicon. For example, Figure 5A shows a single peak for exon 17b of CFTE

(Cystic Fibrosis Transmembrane Conductance Regulator) gene, whereas the melt curve for an amplicon from exon 7 of CFTR shows two peaks, which could be interpreted as indicative of two separate amplicons (Figure 5B). However, analysis by agarose gel electrophoresis showed only one peak. To solve this conflict, an understanding of how melt curves are produced is needed. It should be emphasized that intercalating dyes used in qPCR, such as SYBR Green, will fluoresce only when the dye is bound to dsDNA, but not in the presence of a ssDNA, or when the DNA is free in solution. After the amplification cycle in qPCR, the instrument starts at a preset temperature above the primer T_m , and as the temperature increases dsDNA denatures becoming ssDNA and the

Figure 3A: Gene expression for stool samples taken from 60 patients. The condition of the patient is indicated by the bottom row of the panel and by the type of line. There were 20 normal patients (gray lines), 10 with IBD (dashed lines), and 30 with cancer (black lines). Instances of high expression appear on the right and those with low expression on the left. Expression was measured by CP and scales were chosen so that minimum values line up on the Min mark labeled at the top of the panel. The same is true for the maximum values which line up under the mark labeled Max.

Figure 3 B: This panel displays gene expression for stool samples taken from 30 cancer patients. Stage of cancer is indicated by the bottom row of the panel and by the type of line. There were 20 patients with stage 0 or 1 (gray lines), 5 with stage 2 (dashed lines), and 5 with stage 3 (black lines) cancer. The 30 noncancerous patients (stage NA) are not shown. C) Gene expression for tissue samples taken from 60 patients. Conditions of the patient are the same as in panel A. D) This panel displays gene expression for tissue samples taken from 30 cancer patients. Stages of cancer are indicated as in panel B.

Figure 4A: Graph illustrating the relation between fluorescence and temperature for labeled probe designed for a wild type (Wt) sequence, homozygous Wt, heterozygous (Wt/Mut), and homozygous mutant (Mut/Mut) combination.

Figure 4 B: Graph of the first derivative of melting curve ($-dF/dT$) that pinpoints the temperature of dissociation, defined as 50% dissociation, by formed peaks. Figure courtesy of Integrated DNA Technologies (www.idtdna.com); Downey N. (2014) *Interpreting melt curves: An indicator, not a diagnosis*. [Online] Coralville, Integrated DNA Technologies. [Accessed May, 26, 2017]

Figure 5A: Melt curves from qPCR of the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene. A. An amplicon from CFTR exon 17b reveals a single peak following melt curve analysis.

Figure 5B: An amplicon from CFTR exon 7 reveals two peaks. Figure courtesy of Integrated DNA Technologies (www.idtdna.com); Downey N. (2014) *Interpreting melt curves: An indicator, not a diagnosis*. [Online] Coralville, Integrated DNA Technologies. [Accessed May, 26, 2017]

dye therefore dissociates from the ssDNA (Figure 3A). The change in slope of this curve when blotted as a function of temperature to obtain a melt curve for CFTR exon 17b (Figure 4A). However, if we allow for the possibility that DNA may assume an intermediate state that is neither dsDNA or ssDNA, raw data from CFTR exon 7 melt will look Figure 4B. This could happen when there are regions of the amplicon that are more stable (e.g., G/C rich), which do not melt immediately, but maintain their ds configuration until the temperature becomes sufficiently high to melt it, which results in two phases (Figure 4B). Additional sequence factors, such as amplicon misalignment in A/T rich regions, and designs that have secondary structure in the amplicon region, can also produce products that melt in multiple phases.

An advancement of MCA, referred to as High Resolution Melt (HRM), discovered and developed by Idaho Technology and the University of Utah [66, http://www.dna.utah.edu/Hi-Res/TOP_Hi-Res%20Melting.html], which has been useful for mutation detection and SNPs, enabling differentiation of homozygous wildtype, heterozygous and homozygous mutant alleles from the dissociation patterns. HRM has been used to identify variation in nucleic acid sequences, enabled by use of a more advanced software, and is therefore less expensive than probe-based genotyping methods, and allows for identification of variants quickly and accurately [67]. This method has been widely used in molecular diagnosis and for detection of mutations [68-74].

MCA is an assessment of dissociation characteristics of dsDNA during heating, leading to rise in absorbance, intensity and hyperchromicity. The temperature at which 50% of DNA is denatured is referred to as melting point, T_m .

Gathered information can be used to infer the presence of single nucleotide polymorphism (SNP), as well as clues to

molecule's mode of interaction with DNA, such as intercalator slots in between base pairs through pi stacking and increasing salt concentration, leading to rise in melt temperature, whereas pH can affect DNA's stability, leading to lowering of its melting temperature [75,76]. Originally, strand dissociation was measured using UV absorbency, but now techniques based on fluorescence measurements using DNA intercalating fluorophores such as SYBR Green I, Eva Green, or Fluorophore-labelled DNA probes (FRET probes) when they are bound to ds DNA [75] are now common. Specialized thermal cyclers that run the qPCR, such as Roche Light Cycler (LC) 480®, used in this study, is programmed to produce the melt curve after the amplification cycles are completed. As the temperature increases, dsDNA denatures becoming ss and the dye dissociates, resulting in decrease in fluorescence. The graph of the negative first derivative of the melting-curve (-dF/dT) represents the rate of change of fluorescence in the amplification reaction, and allows pin-pointing the temperature of dissociation (50% dissociation) using formed peaks to obviate or complement sequencing efforts [77].

The melting temperature (T_m) of each product is defined as the temperature at which the corresponding peak maximum occurs. The MCA confirms the specificity of the chosen primers, as well as reveals the presence of primer-dimers, which usually melt at lower temperatures than the desired product, because of their small size, and their presence severely reduce the amplification efficiency of the target gene as they compete for reaction components during amplification, and ultimately the accuracy of the data. The greatest effect is observed at the lowest concentrations of DNA, which ultimately compromises the dynamic range. Moreover, nonspecific amplifications may result in PCR products that melt at temperatures above or below that of the desired product. Optimizing reaction components (Mg^{2+} , detergents, SYBR Green I concentration) and annealing temperatures aid in decreasing nonspecific product formation [78-80]. Adequate product design, however, is considered to be the best method to avoid nonspecific products' formation. Including a negative control will determine if there is a co amplified genomic DNA [81,82]. The formula for T_m calculation is shown by the equation:

$$T_m = \frac{\sum \Delta H_{on-n}}{RT \ln C_t} + 273.15, \sum \Delta S_{on-n} + R \ln C_t$$

where thermodynamic parameter ΔH_o is Enthalpy changes, ΔS_o parameter is Entropy changes, and C_t is total strand concentration; these free-energy parameters predict T_m of most oligonucleotide duplexes to within 5°C; and permit prediction of DNA, as well as RNA duplex stabilities. It should be noted that T_m depends on the conditions of the experiment, such as oligonucleotide concentration, salts' concentration, mismatches and single nucleotide polymorphisms (SNPs) [83]. OligoAnalyzer® Tool [www.idtdna.com/analyzer/Applications/Oligoanalyzer] allows for calculating the T_m of employed nucleotides.

Statistical and bioinformatics analysis

Gene expressions were standardized by dividing the SNORD48 value while raw melting temperatures were used. Analysis were done using the software R (version 3.1.3), with the package MASS [84]. One individual had so many missing values that this case was not used in the analysis so that the number of individuals is 24. For each standardized gene and each melting temperature, a one way ANOVA was used to obtain a p-value. There were four levels of the explanatory variable: Control, Soby, Pom, and Both. Parallel coordinate plots (parcoord command in R) [85] were used to visualize the data for each gene and each melting temperature. Coordinates were ordered using the magnitude of the p-value. The two sample t-test was used on gene expression to compare control to sobya and control to both (t.test command in R with var.equal=FALSE) [86]. P-values were adjusted to control for false discovery rate. The method is outlined in [87] Benjamini and Yekutieli (p.adjust command in R with method='BY').

We have bioinformatically correlated the 2-7 or 2-8 complement nucleotide bases in the mature miRNAs with the untranslated 3' region of target mRNA (3' UTR) of a message using a basic algorithm such as Broad's Institute's Target Scan [88] <http://www.targetscan.org/archives.html>, which provides a precompiled list for their prediction.

Results

At base line, all participants in the trial excreted urinary total polyphenols; however, the inter individual variation was considerably high (4.89-12.59 mg GAE/100 ml urine).

Composition of the three supplements (FS, PGJ and FS + PGJ) served to the volunteers is presented in Table 1. The initial and final mean urinary polyphenols, plasma and urinary ant oxidative activity, urinary TBARS and erythrocytic GST, as well as the daily portion of PGJ provided 21 mg PP /day, and the combination of PGJ – FS was 9 mg PP /day, as presented in Table 2.

Figure 2 is a layout of RT² miRNA PCR Array Human Cancer microRNA (MAH-102A). Figure 6 is a graphical representation of the parallel plot coordinates of the studied miRNA genes for melting temperature curve analysis. The genes were ordered using the p-values of a one way ANOVA based on groups. Genes with the smallest p-values are presented first. Figures 3 through 5 represent characteristics of melt curve analysis protocols.

Figure 6a show eight employed control genes (Snord48, Snord47, Snord44, RNU6-2, MiRTC1, miRTC2, and PPC1, PPC2). In Figure 6b, five miRNA genes (miR-184, miR-203, miR-124, miR-96 and miR-378) show clear separation. Gene miR-184 has the highest separation from the control gene. MiR-203 genes are hardly amplified in Soby, while it is highly expressed in Pomegranate. For miR-373 gene, the control group is different from the other three treatment groups. For genes miR-124, miR-96 and miR-378, Pomegranate is well separated from other three groups. In Figure 6c, for gene miR-301a, the control is separated from the other three groups. Additional miRNA genes are not

shown, as their p-values are greater (less significant), and the graphs did not show any meaningful separations.

Bioinformatics analysis using the Target Scan algorithm [88] for up-regulated and down-regulated mRNAs genes is shown in Table 3. The program yielded 21 mRNA genes encoding different cell regulatory functions. The first 12 of these mRNAs were found with the DAVID program [89] to be active in the nucleus and related to transcriptional control of gene regulation. For down regulated miRNAs, the DAVID algorithm found the first four of these mRNAs to be clustered in cell cycle regulation categories. [90].

Discussion

Suitability of stool as a medium for developing a sensitive molecular biomarker screen

Stool represents a challenging environment, as it contains many substances that may not be consistently removed in PCR, in addition to the existence of certain inhibitors [91-94], which all must be removed for a successful PCR reaction. Our results [56, 75, 78, 95-97] and others [9] have shown that the presence of non-transformed RNA and other substances in stool do not interfere with measuring miRNA expressions, because of the use of suitable PCR primers, and the robustness of the real-time qPCR method. Besides, stool colonocytes contain much more miRNA and mRNA than that available in free circulation, as in plasma [97, 98], all factors that facilitate accurate and quantitative measurements.

PCR amplification and the effect of Inhibitory Substances

PCR has been used for miRNAs quantification because of its extreme sensitivity. This method, however, could lead to errors because of the presence of inhibiting substances, representing diverse compounds with different properties and mechanisms of action, which induce their effects by direct interaction with DNA that will be amplified, or through interference with the employed thermos table DNA polymerase. Agents that reduce Mg²⁺ availability, or interfere with the binding of Mg²⁺ to DNA polymerase could inhibit the PCR reaction [92]. Calcium ion is another inorganic inhibiting substance, although most PCR inhibitors are organic compounds (e.g., bile salts, sodium dodecyl sulphate, urea, phenol, ethanol, polysaccharides), as well as proteins (e.g., collagen, hemoglobin, immunoglobulin G and proteinase) [99]. The existence of polysaccharides in stool could decrease the capacity to resuspend precipitated RNA, or disrupt the enzymatic reaction by mimicking the structure of nucleic acid. The DNA template of the PCR, as well as primers binding to DNA template can be inhibited by nucleases and other inhibitors, [93]. Remedial strategies for removal of inhibitors in stool, such as additional extraction steps, sephadex G-200 chromatography, heat treatment before the PCR, chloroform extraction, treatment with activated carbon, adding BSA, or dilution of sample, have been suggested [94]. We found the dilution method, in which the extracted ribonucleic acid (RNA) is diluted in the reaction mixture with distilled water or an isotonic buffer, to be the most practical method for preventing PCR inhibition using a commercially available diluent [100-102].

Melting curve analysis (MCA)

Microscale thermophoresis is a method that determines the stability, length, conformation and modifications of DNA and RNA. It relies on the directed movement of molecules in a temperature gradient that depends on surface characteristics of the molecule, such as size, charge and hydrophobicity. By measuring thermophoresis of nucleic acids over a temperature gradient,

Table 3: Up-regulated and down-regulated target mRNA genes detected by a DAVID Bioinformatics algorithm.

Up-regulated target mRNA genes

BCL11B, CUGBP2, EGR3, DLHAP2, NUFIP2, KLF3, MECP2, ZNF532, APPLI1, NFIB, SMAD7, SNF1LK, ANKRD52, C17orf39, FAM13A1, GLT8D3, KIAA0240, PCT, SOCS6, TNRC6B and UHRF1BP1.

Down-regulated target mRNA genes

TGFB1, CKS2, IGF2, KLK10, FLNA, CSE1L, CXCL3, DPEP1 AND GUCA2B

one finds clear melting transitions, and can resolve intermediate conformational states (Figure 3). These intermediate states are indicated by an additional peak in the thermophoretic signal preceding most melting transitions (Figure 3B) [73, 77-79]. Agarose gel visualization is the gold standard for analyzing PCR products. Alternatively to reduce the number of gels needed to conform the presence of a single amplicon, "uMelt" melting curve prediction software (<http://www.dna.utah/umelt/umelt.html>) can be used to confirm that a single amplicon is generated by PCR [80]. This program predicts melt curves and their derivatives for qPCR-length amplicons, and is suited to test for multiple peaks in a single amplicon product.

...Because SYBR Green I dye has several limitations, including inhibition of PCR, preferential binding to CG-rich sequences and effects on MCA, two intercalating dyes SYTO-13 and SYTO-82 were tried and did not show these negative effects, and SYTO-82 demonstrated a 50-fold lower detection limit [81], as well as best combinations of time-to threshold (Tt) and signal-to-noise ratio (SNR) [82]. To optimize performance of the buffer, a PCR mix supplemented with two additives, 1M 1, 2-propanediol and 0.2 M trehalose, were shown to decrease Tm, efficiently neutralize

PCR inhibitors, and increase the robustness and performance of qPCR with short amplicons [83]. "UAnalyzeSM" is another web-based tool, similar to uMELT, for analyzing high-resolution melting PCR products' data, in which recursive nearest neighbor thermodynamic calculations are used to predict a melt curve. Using 14 amplicons of CYBB [cytochrome b-245 heavy chain, also known as cytochromae b(558) subunit], the main +/- standard deviation, the difference between experimental and predicted fluorescence at 50% helicity was -0.04 +/- 0.48oC [64].

In our study, we found the melt curve analysis to be a useful and an informative method because after the statistical analysis carried on our miRNA expression samples showed no preferential expression of any of the 88 miRNA genes, a melt curve analysis on the same samples found that we could distinguish 7 miRNA (miR-184, miR-203, miR-373, miR-124, miR-96, miR-373 and miR-301a), due to different separation melting profiles (Figure 6). Thus, we believe that it is imperatives for investigators to run this kind of analysis on samples that particularly may not show expression differences in their mRNA or miRNA studied genes, such as nutritional samples.

Chart 1. A chart illustrating the design of the randomized controlled trial.

Figure 7: A graphical representation of the parallel plot coordinates of the studies miRNA genes. The genes were ordered using the p-values of a one way ANOVA based on groups. Genes with the smallest p-values are presented first.

Figure 7a: show control genes. In

Figures 7b, c: five miRNA genes show separation.

Bioinformatics methods to correlate seed miRNA Data with messenger (m) RNA Data

To provide information about complex regulatory elements, we correlated miRNA results with our available mRNA data [1], as well as those data available in the open literature using computer model Target Scan [88, 89]. The authenticity of functional miRNA/mRNA target pair, once identified was validated by fulfilling four basic criteria: a) miRNA/mRNA target interaction can be verified, b) the predicted miRNA and mRNA target genes are co-expressed, c) a given miRNA must have a predictable effect on target protein expression, and d) miRNA-mediated regulation of target gene expression should equate to altered biological function. Bioinformatics showed 21 up regulated mRNA genes encoding different cell regulatory functions, and 12 of these mRNAs were found to be active in the nucleus and related to transcriptional control of gene regulation. For down-regulated miRNAs, four of the mRNAs appeared to be clustered in cell cycle regulation categories (Table 3) [56].

Clinical Significance

The clinical significance of the study presented above is that using melting temperatures for analyzing nutrient-gene data is a promising new approach for identifying key regulatory miRNA genes related to metabolites rich in polyphenols, Probiotic lactobacilli, or combinations of the two metabolites. Melt curve analysis is a powerful novel approach because after the statistical analysis carried on our miRNA samples produced negative gene expression (Cq) results, running melt curve analysis on the same samples identified 7 of the 88 miRNA genes imprinted on the highly sensitive focused PCR arrays (~ 8% of the genes), and using parallel coordinates plots showed noticeable separation of melt curve profiles. Thus, we believe that it is imperative for investigators to run this kind of MCA on nutrition samples that are mild in nature, and many not normally show significant differences in the expression of studied miRNA genes, particularly at the early stages (0-1) of the cancer, before metastasis, so the cancer can be cured. The same analysis can also be envisioned for messenger mRNA amplifications, using mRNA arrays, and then using bioinformatics resources to correlate mRNA with miRNA data.

We are also planning to validate these initial results by carrying out additional miRNA nutrigenomic expression studies, with much more observations using PP, FS and their combinations, and collectively the obtained results would fully demonstrate the sensitivity/specificity of this powerful systemic molecular approach for analyzing nutrient-gene data.

Research efforts for the management of cancer are directed to identify new strategies for its early detection. Stable miRNAs are a new promising class of circulating biomarkers for cancer detection. However, the lack of consensus on data normalization, using relative PCR quantification methods, has affected the diagnostic potential of circulating miRNAs. There is thus a growing interest in techniques that allow for an absolute quantification

of miRNAs, which would be more precise, and therefore more useful for early diagnosis of this curable cancer, if the cancer can be detected at the early premalignant disease stage (stage 0-1).

Recently, digital PCR, mainly based on droplets generation, emerged as an affordable technology for the precise and absolute quantification of nucleic acids (103, 1'04). Given its reproducibility and reliability, as chip-digital absolute PCR quantification technique has become more established, it would be a robust tool for the quantitative assessment of miRNA copy number necessary for the diagnosis of the cancer, as well as for the identification and quantification of miRNAs in other biological samples such as circulating exosomes or protein complexes.

Acknowledgments

We express our deep thanks to those volunteers who participated in this research by completing a dietary questionnaire, and providing urine, blood and stool samples for the study. We thank Dr. Clark D. Jeffries at Renaissance Computing Institute, University of North Carolina at Chapel Hill for his bioinformatics analysis, Funds for this research were provided by NIH Grant 1R43CA144823, and by GEM Tox Labs operating funds.

References

1. Ahmed FE, Vos P, James S, Lysle DT, Allison RR, Flake G, Naziri W, et al. Transcriptomic molecular markers for screening human colon cancer in stool and tissue. *Cancer Genom Proteom*. 2007;4(1):1-20.
2. Ahmed FE. Microarray RNA transcriptional profiling: Part I. Platforms, experimental design and standardization. *Exp Rev Mol Diag*. 2006;6(4):535-550.
3. Ahmed FE. Microarray RNA transcriptional profiling: Part II. Analytical considerations and annotations. *Exp Rev Mol Diag*. 2006;6(5):703-715.
4. Lee RC, Feinbaum RL and Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes a small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993;75(5):843-854.
5. Kozomara A and Griffiths JS. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42(Database issue):D68-73. doi. 10.1093/nar/gkt1181
6. Lund E and Dahlberg JE. Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. *Cold Spring Harb Symp Quant Biol*. 2006;71:59-66.
7. Lim LP, Lau NC, Garrett-Engle P, Grimson A, Schelter JM, Castle J, Bartel DP A, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005;433(7027):769-773.
8. De Caterina R and Madonna R. Nutrients and gene expression. *World Rev Nutr Diet*. 2004;93:99-133.
9. Link A, Balaguer F, Shen Y, Nagasaka T, Lozano JJ, Boland CR, Goel A., et al. Fecal MicroRNAs as novel biomarkers for colon cancer screening. *Cancer Epidemiol Biomarkers Prev*. 2010;19(7):1766-1774. doi. 10.1158/1055-9965
10. Biomarkers Definition Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Therapeutics*. 2001;69(3):89-95.

11. Strimbu K and Tavel JA. What are biomarkers?. *Curr Opin HIV AIDS*. 2010;5(6):463-466.
12. Roever L. Endpoints in Clinical Trials: Advantages and Milestones. *Evidence Based Medicine and Practice*. 2016;1:2. doi: 10.4172/ebmp.1000e111
13. Endpoints: How the Results of Clinical Trials are Measured. 2004.
14. Clinical endpoints: Principles of Translational Science in Medicine. 2nd Ed. 2015.
15. Bautista RR, Gomez AO, Miranda AH, Dehesa AZ, Villarreal-Gmza C, Avila-Moreno F, Arrieta, et al. Long-coding RNAs: Implications in targeted diagnosis, prognosis, and improved therapeutic strategies in human non- and triple-negative breast cancer. *Clin Epigenet*. 2018;10(88). doi: 10.1186/s13148-018-0514-z
16. Klasić M, Markulin D, Vojta A, Samaržija I, Biruš I, Dobrinčić P, Ventham NT, et al. Promoter methylation of the MGAT3 and BACH2 genes correlates with the composition of the immunoglobulin G glycome in inflammatory bowel disease. *Clin Epigenetics*. 2018;10(75):1-14.
17. Research (CBER). Guidance for industry: Clinical trial endpoints for the approval and of cancer drugs and biologics. 2007.
18. Werner RM and Pearson TA. LDL-Cholesterol. A risk factor for Coronary Artery Disease from Epidemiology to Clin Trials. *Canad J Cardiology*. 1998;14 Suppl B.3B-10B.
19. Bikdeli B, Pumanithinot N, Akram Y, Lee L, Desai NR, Ross J, Krumholz HM, et al. Two decades of cardiovascular trials with primary surrogate endpoints 1990-2011. *J Am Heart Assoc*. 2017;6(3):e005285. doi: 10.1161/JAHA.116.005285
20. Waladkhani AR. Conducting clinical trials: A theoretical and practical guide. 2008.
21. Wenting and Sargent DJ. *Statistics and Clinical Trials: Clinical Radiation Oncology*. 3rd Ed. 2012.
22. Hussein L, Abdl-Rehim EA, Afifi AMR, El-Arab AE and Labib E. Effectiveness of apricots (*Prunus ameniea*) juice and lactic acid fermented sobya on plasma levels of lipid profile parameters and total homocysteine among Egyptian adults. *Food Nutr Sci*. 2014; 5(22):11.
23. World Health Organization (WHO). Cardiovascular Diseases (CVDs). WHO Fact Sheet No. 317, Geneva. 2011.
24. Bernedetti S, Catalani S, Palma F and Trari FC. The antioxidant protection of CELLFOOD® against oxidative change in vitro. *Food Chem Toxicol*. 2011;49(9):2292-2298. doi: 10.1016/j.fct.2011.06.029
25. Bengmark S. Advanced glycation and lipoxidation end products--amplifiers of inflammation. the role of food. *J Parenter Enteral Nutr*. 2007;31(5):430-440.
26. Gouda M, Moustafa A, Hussein L, and Hamza M. Three week dietary intervention using apricots, pomegranate juice or/and fermented sour sobya and impact on biomarkers of antioxidative activity, oxidative stress and erythrocytic glutathione transferase activity among adults. *Nutritional J*. 2016;15:52. doi: 10.1186/s12937-016-0173-x
27. Esmailzadeh A, Tahbaz F, Gaieni I, Alavi-Majd H. Cholesterol-lowering effect of concentrated pomegranate juice consumption in Type II diabetic patients with hyperlipidemia. *Int J Vit Nutr Res*. 2006;76(3):147-151.
28. Zhuang G, Liu XM, Zhang QX, Tian F W. Research advances with regards to clinical outcome and potential mechanisms of the cholesterol-lowering effects of probiotics. *Clinical Lipidology*. 2012;7(5):501-50.
29. van Baarlen P, Troost F, van der Meer C, Hooiveld G. Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. *Proc Natl Acad Sci USA*. 2011;108(Suppl 1):4562-4569.
30. Richards AL, Burns MB, Alazizi A, Barreiro LB, Roger Pique-Regi, Ran Blekman. Genetic and transcriptional analysis of human host response to healthy gut microbiota. *MSystems*. 2016;1(4):e00067-16. doi:10.1128/mSystems
31. Noori-Daloi MR and Nejatizadeh A. Nutritional transcriptomics: An Overview. Eds Debasis Bagehi, Anand Swaroop and Manashi Bagehi. 2015:545-556.
32. Luu-The V, Paquet N, Calvo E, Cumps J. Improved real-time RT-PCR measurements using second derivative calculation and double correction. *BioTechniques*. 2005;38(2):287-293.
33. Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the $\Delta\Delta Ct$ method. *Methods*. 2001;25(4):402-408.
34. Reinhart BJ, Slack FJ, Basson M, Pasquinell AE, Bettinger JC, Rougvie AE, et al. RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*. 2000;403(6772):901-906.
35. Tellman G. The E-method: a highly accurate technique for gene-expression analysis. *Nature Methods*. 2006.
36. http://www.lightcycler-online.com/lc_sys/soft_nd.htm#quant.
37. LightCycler. Roche Molecular Biochemicals. Mannheim, Germany. 2001:64-79.
38. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocol*. 2009;4(1):44-57. doi: 10.1038/nprot.2008.211
39. Ririe KM, Rasmussen RP and Wittwer CT. Product determination by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*. 1997;245(2):154-160.
40. Xu P, Guo M and Hay BA. MicroRNAs and the regulation of cell death. *Trend Genet*. 2004;20(12):617-624.
41. Malumbres M. miRNAs and cancer: an epigenetics view. *Mol Aspects Med*. 2013;34(4):863-874. doi: 10.1016/j.mam.2012.06.005
42. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, et al. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell*. 2007;129(2):303-317.
43. Phua YL, Chu JY, Marrone AK, Bodnar AJ, Sims-Lucas S, Ho J. Renal stromal miRNAs is required for normal nephrogenesis and glomerular mesangial survival. *Physiological Reports*. 2015;3(10):pii.e12537. doi:10.14814/phy2.12537
44. Maes OC, Chertkow HM, Wang E and Schipper HM. MicroRNA. Implications for Alzheimer Disease and other Human CNS Disorders. *Current Genomics*. 2009;10(3):154-168. doi: 10.2174/138920209788185252
45. Li J, Li J, Liu X, Qin S, Guan Y, Liu Y, et al. MicroRNA expression profile and functional analysis reveal that miR-382 is a critical novel gene of alcohol addiction. *EMBO Mol Med*. 2013;5(9):1402-1414. doi: 10.1002/emmm.201201900

46. Romao JM, Jin W, Dodson MV, Hausman GJ, Moore SS, Guan LL. MicroRNA regulation in mammalian adipogenesis. *Exp Biol Med*. 2011;236(9):997-1004. doi: 10.1258/ebm.2011.01110
47. Mencía A, Modamio-Høybjør S, Redshaw N, Morín M, Mayo-Merino F, Olavarrieta L, et al. Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. *Nat Genet*. 2009;41(5):609-613. doi: 10.1038/ng.355
48. Hughes AE, Bradley DT, Campbell M, Lechner , Dash DP, Simpson DA, et al. Mutation Altering the miR-184 Seed Region Causes Familial Keratoconus with Cataract. *Am J Hum Genet*. 2011;89(5):628-633. doi: 10.1016/j.ajhg.2011.09.014
49. de Pontual L, Yao E, Callier P, Faivre L, Drouin V, Cariou S, et al. Germline deletion of the miR-7-92 cluster causes kel skeletal and growth defects in humans. *Nat. Genet*. 2011;43(10):1026-1030. doi: 10.1038/ng.915
50. Tuddenham L, Jung JS, Chane-Woon-Ming B, Dölken L, Pfeffer S. Small RNA deep sequencing identifies microRNAs and other small noncoding RNAs from human herpesvirus 6B. *J Virol*. 2012;86(3):1638-1649. doi: 10.1128/JVI.05911-11
51. Lu M, Zhang Q, Deng M, Guo Y, Wei Gao, Qinghua Cui, et al. An analysis of human microRNA and disease association. *Plos One*. 2008;3(10):e3420.
52. Gregory RI and Shiekhattar R. MicroRNA biogenesis and cancer. *Cancer Res*. 2005;65(9):3509-3512.
53. Calin GA, Ferracin M, Cimmino A, Dileva G, Shimizu M, Wojcik SE, Iorio MV, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Eng J Med*. 2005;353(17):1793-1801.
54. Chang-Zheng C. MicroRNAs as oncogenes and tumor suppressors. *N Eng J Med*. 2005;353(17):1768-1771.
55. Calin GA, Sevignai C, Dumitru CD, Hyslop T. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA*. 2004;101(9):2999-3004.
56. Schepler T, Reinert JT, Oslensfeld MS, Christensen LL , Silahatoglu AN, Dyrskjøt L, Wiuf C, et al. Diagnostic and prognostic microRNAs in Stage II colon cancer. *Cancer Res*. 2008;68(15):6416-6424. doi:10.1158/0008-5472.CAN-07-6110
57. Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Elise D. Bowman, Nozomu Yanaihara, Siu Tsan Yuen, et al. MicroRNA expression profile associated with progression and therapeutic outcome in colon adenocarcinoma. *J Am Med Assoc*. 2008;299(4):425-436. doi: 10.1001/jama.299.4.425
58. Calin GA and Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006;6(11):857-866.
59. Ahmed FE, Ahmed NC, Vos PW, Bonnerup C, Atkins JN, Casey M, Nuovo GJ, et al. Diagnostic microRNA markers to screen for sporadic human colon cancer in stool. I. Proof of principle. *Cancer Genom Proteom*. 2013;10(3):93-113.
60. Varga A and Delano J. Effect of amplicon size, melt rate, and dye translocation. *J Virol Meth*. 2006;132(1-2):146-153.
61. Mendes RE, Kiyota KA, Monteiro J, Castanheira M, Andrade SS, Gales AC, et al. Rapid detection and identification of metallo-β-Lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. *J Clin Microbiol*. 2007;45(2):544-547.
62. Guion CE, Ochoa TJ, Walker CM, Barletta F, Cleary TG. Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis and real-time multiplex PCR. *J Clin Microbiol*. 2008;46(5):1752-1757. doi: 10.1128/JCM.02341-07
63. Winder L, Phillips C, Richards N, Ochoa-Corona F, Hardwick S, Vink CJ, et al. Evaluation of DNA melting analysis as a tool for species identification. *Meth Ecology Evol*. 2011;2(3):312-320.
64. Von Keyserling H, Bergmann T, Wiesel M, Kaufmann AM. The use of melting curves as a novel approach for Validation of real-time PCR instruments. *BioTechniques*. 2011;51(3):179-184. doi: 10.2144/000113735
65. Bohling SD, Wittwer CT, King TC, Elenitoba-Johnson KS. Fluorescence melting curve analysis for the detection of the bcl-1/JH translocation in mantle cell lymphoma. *Lab Invest*. 1999;79(3):337-345.
66. de Fdippes FF, Wang J-W and Weigel D: MIGS: miRNA-induced gene silencing. *The Plant J* 70(3): 541-547, 2011.
67. Dwight Z, Palais R and Wittwer CT. μMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application. *Bioinformatics*. 2011;27(7):1019-1020. doi: 10.1093/bioinformatics/btr065
68. Downey N. Interpreting melt curves. An indicator, not a diagnosis.
69. Farrar JS, Reed GH, Wittwer CT. High-resolution melting curve analysis for molecular diagnosis. In *Molecular Diagnostics*. 2014;229-245. doi: 10.1016/B978-0-12-374537-8.00015-8
70. Krypuy M, Ahmed AA, Etemadmoghadam D, DeFazio A, Fox SB, Brenton JD, et al. High resolution melting for mutation scanning of TP53 exon 5-8. *BMC Cancer*. 2007;7:168.
71. Pasay C, Arlian L, Morgan M, et al. High -resolution melt analysis for the detection of a mutation associated with permethrin resistance in a population of scabies mites. *Med Vet Entomol*. 2008; 22(1):82-88.
72. Montgomery JL, Sanford LN, Twitter CT. High-resolution DNA melting analysis in clinical research and diagnosis. *Expert Rev Mol Diagn*. 2010;10(2):219-240. doi: 10.1586/erm.09.84
73. Ansevin AT, Vizard DL, Brown BW and McConathy J. High-resolution thermal denaturation of DNA. I. Theoretical and practical considerations for the resolution of thermal subtransitions. *Biopolymers*. 1976;15(1):153-174. doi:10.1002/bip.1976.360150111
74. Ririe KM, Rasmussen RP and Wittwer C T. Product differentiation by analysis of DNA during melt curves during the polymerase chain reaction. *Anal Biochem*. 1997;245(2):154-160.
75. Wittwer CT. High-resolution DNA melting analysis: Advancements and limitations. *Hum Mutat*. 2009;30(6):857-9. doi: 10.1002/hum.u.20951
76. Ahmed FE, Hussein LA, Gouda MM, Vos PW and Ahmed NC. Melt Curve Analysis in Interpretation of Nutrigenomics' MicroRNA Expression Data. *Trends Res*. 2018;1 (1):1-10.
77. Ahmed FE, Gouda MM, Hussein LA, Ahmed NC, Vos PW, Mohammad M. Role of Melt Curve Analysis in Interpretation of Nutrigenomics' MicroRNA Expression Data. *Cancer Genom. Proteom*. 2017;14(6):469-481.
78. Freier SM, Kierzek R, Jaeger JA, Sugimoto N M H Caruthers, T Neilson, D H Turner, et al. Improved free-energy parameters for predictions

- of RNA duplex stability. *Proc Natl Acad Sci USA*. 1986;83(24):9373-9377.
79. Lay MJ and Wittwer CT. Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clin Chem*. 1997;43(12):2262-2267.
80. Wienken C J, Baaske P, Duhr S, Braun D. Thermophoretic melting curve quantify the conformation and stability of RNA and DNA. *Nucleic Acids Res*. 2011;39(8):e52. doi: 10.1093/nar/gkr035
81. Dwight Z, Palais R and Wittwer CT. μ MELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application. *Bioinformatics*. 2011;27(7):1019-1020. doi: 10.1093/bioinformatics/btr065
82. Gudnason H, Dufva M, Bang D D, Wolff A. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. *Nucleic Acids Res*. 2007;35(19):e127. doi: 10.1093/nar/gkm671
83. Oscorbin IP, Belousova EA, Zakabunin AI, Boyarskikh UA and Filipenko M L. Comparison of fluorescent intercalating dyes for quantitative loop-mediated isothermal amplification. *BioTechniques*. 2016;61(1):20-25. doi: 10.2144/000114432
84. Horakova H, Polakovcova I, Shaik GM, Eitler J, Bugajev V, Draberova L, Draber P, et al. 1,2-propanediol-trehalose mixture as a potent quantitative real-time PCR enhancer. *MBC Biotechnol*. 2011;11:41
85. Venables WN and Ripley BD (Eds.). *Modern and Applied Statistics*. Fourth Edition. Springer, New York. 2002.
86. R Core Team R. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Australia. 2015.
87. Ahmed F E. Statistical Analysis of microRNA as markers for Screening of Colon Cancer. *Biostatistics and Biometrics J (BBOAJ)*. 2018;6(4):1-4. doi: 10.19080/BBOAJ.2018.06.555691
88. Benjamini Y and Yekutieli D. The control of the false discovery rate in multiple testing under dependency. *Annals Stat*. 2001;29(4):1165-1188.
89. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocol*. 2009;4(1):44-57. doi: 10.1038/nprot.2008.211
90. Thompson S M, Ufkin JA, Sathyanarayana M L, Liaw P, et al. Common features of micro RNA target prediction tools. *Front Genet*. 2014; 5:23. doi: 10.3389/fgene.2014.00023
91. Al-Soud WA and Radstrom P. Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl Env Microbiol*. 1998;64(10):3748-3753.
92. Wilson I G. Inhibition and facilitation of nucleic acid amplification. *Appl Env Microbiol*. 1997;63(10):3741-3751.
93. Montiero I, Bonnemaïson D, Vekris A, Petry KG, J Bonnet, R Vidal, et al. Complex polysaccharides as PCR inhibitors in feces. *Helicobacter pylori model*. *J Clin Microbiol*. 1997;35(4):995-998.
94. Schrader C, Schielke A, Ellerbroek L and Johne R. PCR inhibitors- occurrence, properties and removal. *J Appl Microbiol*. 2012;113(5):1014-1026. doi: 10.1111/j.1365-2672.2012.05384.x.
95. Ahmed FE, Ahmed NC, Vos P, Bonnerup C, Atkins JN, Casey M. Diagnostic microRNA markers to screen for sporadic human colon cancer in blood. *Cancer Genom Proteom*. 2012;9(4):179-192.
96. Ahmed F E, Ahmed N C, Gouda M and Bonnerup C. MicroRNAs as Molecular Markers for Screening of Colon Cancer. *Case Rep Surg Invasive Proced*. 2017;1(2):14-17.
97. Ahmed F E, Ahmed N C, Gouda M and Vos P W. MiRNAs for the Diagnostic Screening of Early Stages of Colon Cancer in Stool or Blood. *Surgical Case Reports and Reviews*. 2017;1(1):1-19. doi: 10.5761/SCRR.1000103
98. Ahmed F E, Vos P W, Ijames S, Lysle DT, Flake G, Sinar DR, Naziri W, et al. Standardization for transcriptomic molecular markers to screen human colon cancer. *Cancer Genom Proteom*. 2007; 4(6): 419-431.
99. Davidson LA, Lupton JR, Miskovsky E, Fields AP. Quantification of human intestinal gene expression profiling using exfoliated colonocytes. a pilot study. *Biomarkers*. 2003;8(1):51-61.
100. Rådström P, Knutsson R, Wolfs P, Lovenklev M. Pre-PCR processing strategies to generate PCR-compatible samples. *Mol Biotechnol*. 2004;26(2):133-146.
101. Scipioni A, Mauroy A, Ziant D, Saegerman C et al. A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition. *Virology J*. 2008;5:94. doi: 10.1186/1743-422X-5-94
102. Ahmed F E. MicroRNAs as Molecular Markers for Colon Cancer Diagnostic Screening in Stool & Blood. *Int Med Rev*. 2017;9:124. doi:10.3390/cancers9090124
103. Ahmed FE. Use of Chip-Based PCR for 3D Absolute Digital Quantification of microRNAs Molecules for The Non-Invasive Diagnostic Screening of Human Colon Cancer in Stool. *Integrative Care Sci Therapeutics*. 2018;5(2):1-1.
104. Ahmed, F E, Gouda M M, Ahmed N C. Chip-Based Digital PCR for Absolute Quantification of Colon Cancer miRNAs with 3D digital, chip-based PCR. *Arch Oncol Cancer Ther*. 2018;1(1):1-24.