Folate and vitamin D: The role of nutritional status and nutrigenetics in predicting levels of extracellular microRNA and circulating DNA methylation status

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Thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy in Food Science

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Statement of Originality

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository**, subject to the provisions of the Copyright Act 1968.

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Ms Emma Beckett
Statement of collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of this thesis a statement clearly outlining the extent of the collaboration, with whom and under what auspices.

Chapter 2: Circulating tumour-suppressor and oncogenic microRNA: The relationships to dietary intake, methyl-donor biochemistry and vitamin D status, implications for use as a biomarker of adenomatous colon polyps, and Chapter 3: Polymorphisms in vitamin D receptor and key folate metabolism enzyme genes: Relationship between risk for adenomatous polyps and plasma expression of selected tumour-suppressor and oncogenic microRNA.

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A/Prof Martin Veysey  Supervisor; involved in study design and oversight; coordinated colonoscopy collection and diagnosis.

Dr Zoe Yates  Supervisor; involved in study design and oversight; involved in collection of blood samples and patient interviews; oversaw genetic analysis.

Dr Konsta Duesing  Supervisor; involved in study design and oversight.

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Chapter 4: Folate and vitamin D stimulation of malignant cell lines; a potential role for DNA methylation in the modulation of microRNA expression.

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Dr Konsta Duesing  Supervisor; involved in study design and oversight.

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Chapter 5: DNA methylation status of CpG Islands of the vitamin D receptor and vitamin D metabolism genes; relationships to genotype, methyl-group diet, plasma vitamin D status and selected systemic circulating microRNA.

Ms Emma Beckett  Plasma 25(OH)D assays; DNA isolation and DNA methylation assays; statistical analysis; miRNA isolation and assays; study design; homocysteine assays; quality control and data cleaning, vitamin D and folate intake in food frequency questionnaires (adenomatous polyp case-control cohort); folate intake in food frequency questionnaires (RHLS cohort).

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Dr Katrina King  Involved in data collection and management (RHLS cohort).

Dr Suzanne Niblett  Involved in data collection and management (RHLS cohort).

Dr John Furst  Supervised collection and analysis of solar irradiance data.

Ms Patrice Jones  Collection and analysis of solar irradiance data.

Funding Sources  CSIRO and ARC.

Ms Emma Beckett  A/Prof Mark Lucock
Statement of authorship

I hereby certify that the work embodied in this thesis contains published papers of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

Ms Emma Beckett

Chapter 1 is based partly on two published review articles. Chapters 2, 3 and 5 are each partly formed by portions of data contained within the 2 published and 1 submitted papers. The details of these manuscripts listed below. Contributions of the candidate as an author are listed following each article below. Articles are included in full as appendices as listed and full citations are included at the commencement of each chapter.

Review Articles

Beckett EL, Yates Z, Veysey M, Duesing K, Lucock M. “The role of vitamins and minerals in modulating the expression of microRNA”. Nutrition Research Reviews. 2014 Jun;27(1):94-106. doi: 10.1017/S0954422414000043 (Appendix A) – EL Beckett researched and prepared the manuscript under the supervision of the co-authors.


Original Research Articles

Beckett EL, Martin C, Choi JH, King K, Niblett S, Boyd L, Duesing K, Yates Z, Veysey M, Lucock M. “Folate status, folate-related genes and serum miR-21 expression: Implications for miR-21 as a biomarker.” BBA Clinical, 2015 Jul 7;4:45-51. doi: 10.1016/j.bbacli.2015.06.006. (Appendix C) – EL Beckett was responsible for the design of the study, data collection, statistical analysis, and preparation of the manuscript. Co-authors supervised this work, were involved in the original cohort design and/or provided data to be used in the analysis in this manuscript.

Beckett EL, Le Gras K, Martin C, Boyd L, Ng X, Duesing K, Yates Z, Veysey M, Lucock M. “Vitamin D receptor polymorphisms relate to risk of adenomatous polyps in a sex specific
manner”, *Nutrition and Cancer*, 2016, Feb-Mar;68(2):193-200, doi: 10.1080/01635581.2016.1142584 (Appendix F) – EL Beckett was involved in the design of the study, data collection and, was responsible for statistical analysis and manuscript preparation. Co-authors supervised this work, were involved in the original cohort design and/or provided data or laboratory work to be used in the analysis in this manuscript.

**Beckett EL, Duesing K, Martin C, Jones P, Furst J, King K, Niblett S, Yates Z, Veysey M, Lucock M. “Relationship between methylation status of vitamin D-related genes, vitamin D levels, and methyl-donor biochemistry,” *Journal of Nutrition & Intermediary Metabolism*, Volume 6, December, 2016, 8–15, doi:10.1016/j.jnim.2016.04.010 (Appendix G) – EL Beckett was responsible for the design of the study, data collection, statistical analysis and preparation of the manuscript. Co-authors supervised this work, were involved in the original cohort design and/or provided data to be used in the analysis in this manuscript.

I attest to the statements above, A/Prof Mark Lucock

**Conference Abstracts**


**Beckett EL, le Gras KC, Veysey M, Boyd L, Ng X, Yates Z, Duesing K, Lucock M. Vitamin D receptor polymorphism FokI alters risk of adenomatous polyps in Australian males, International

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Appendices


Appendix D: Contains food frequency questionnaires.

Appendix E: Contains additional tables for Chapter 2.


Synopsis

miRNA in systemic circulation are proposed as potential biomarkers for disease diagnosis and prognosis. However, miRNA profiles may also be modulated by other exposures such as nutritional status, and this may have consequences for use of miRNA as biomarkers, particularly in diseases for which diet is a modifiable determinant. Furthermore, little is known about the interactions that exist between these relationships and underlying variance in genes related to the processing of nutrients that may influence these relationships, or how these miRNA interact with other modifiers of gene expression, such as DNA methylation.

This thesis focuses on folate and vitamin D, two key micronutrients known to have the potential to influence gene expression. The data presented here investigates the relationships between these micronutrients and related nutrigenetics in predicting levels of extracellular miRNA and circulating DNA methylation status. The studies presented here were designed to capitalise on the availability of two well-characterised human cohorts; a case-control cohort of adenomatous polyp patients and healthy controls (n=263), and an elderly cross-sectional cohort (n=649). These are appropriate cohorts in which to investigate these relationships, as systemic circulating miRNA have been proposed as biomarkers for adenomatous polyps and colorectal cancer (CRC), diseases with known dietary modifiers of risk (including folate and vitamin D) which accumulate over a lifetime of exposures. Four candidate miRNA (let-7a, miR-15a, miR-21 and miR-155) were selected due to a combination of factors; each has known oncogenic or tumour-suppressor properties and each had existing evidence to suggest potential regulation by nutritional factors.

The first results chapter (Chapter 2) presents novel observations on the levels of systemic circulating levels of let-7a, miR-15a and miR-155 in adenomatous polyp cases relative to controls. Furthermore, by adding a sex specific level of analysis, it adds to the body of knowledge surrounding these miRNA and miR-21, which is currently proposed as a biomarker for adenomatous polyps. Novel data on the correlations between blood levels of folate and related micronutrients and the candidate miRNA are presented, with key findings including a positive correlation between red blood cell folate levels and all candidate miRNA, regardless of their tumour-suppressor or oncogenic properties. Stepwise regression analyses investigating the correlations between systemic circulating miRNA levels and multiple dietary intakes, including vitamin D, are also presented.

Chapter 3 builds upon these results by incorporating common folate and vitamin D related genetic polymorphisms into the analyses. The relationships between these polymorphisms, systemic circulating miRNA levels, and risk for adenomatous polyps were assessed, as well as interactions with nutrient status. Statistically significant relationships were identified between multiple
polymorphisms and risk for adenomatous polyps, and miRNA levels, as well as potential interactions between folate status and genotype in predicting miRNA levels. These are the first reported observations of the potential relationships and interactions between miRNA profiles and nutrigenetic variance.

As the human cohorts used can only demonstrate correlation and not causation, Chapter 4 contains data obtained from cell culture models. Three CRC cell lines were used to demonstrate that miRNA are differentially expressed intracellularly and extracellularly under folate excess or deficient conditions, and following stimulation with the active vitamin D metabolite. Treatment with a DNA demethylating agent was also used to demonstrate that some of these processes are dependent on DNA methylation.

The relationships between vitamin D and DNA methylation were further investigated in Chapter 5. A sub-cohort was used to conduct a pilot study investigating the relationships between vitamin D status, methyl donor-related micronutrients and DNA methylation in genes of vitamin D metabolism. The relationship between methylation status in this pathway and the systemic circulating levels of the candidate miRNA were also assessed, and provides new information demonstrating the potential complexity of the complementary pathways for the regulation of cellular processes and pathways.

Together, the data in this thesis constitute a significant contribution to the body of knowledge surrounding the extracellular levels of miRNA, and how this may relate to vitamin D and folate status, related polymorphisms, DNA methylation, and intracellular miRNA expression levels. Relationships were identified between folate status, nutrient intake and systemic circulating levels of multiple candidate miRNA. Relationships identified between polymorphisms in related genes and systemic circulating miRNA levels support these observations, and these observations may link dietary factors to modified risk for disease.

This thesis expands our understanding of how nutrition and nutrigenetics can interact to modify nutrigenomics and disease risk. The data presented here for the candidate miRNA and two key nutrients, provides an impetus to investigate these relationships for other nutrients and miRNA, particularly those known to modify disease risk. These results have implications for the use of systemic circulating miRNA as biomarkers, and may also have implications for the future of personalised nutrition and personalised medicine.
List of Abbreviations

1,25(OH)\textsubscript{2}D – calcitriol
25(OH)D – calcidiol
5-Aza – 5-aza-2’-deoxycytidine
AML – acute myeloid leukaemia
APC – adenomatous polyposis coli
Bcl2 – B-cell lymphoma 2 (Bcl2)
BMI – body mass index
Bmi-1 – B lymphoma mouse Moloney leukemia virus insertion region
bp – base pair
cDNA – copy DNA
C/EBPB – CCAAT/enhancer binding protein beta
C. elegans – Caenorhabditis elegans
CEU – Centre d’Etude du Polymorphisme Humain; Utah Residents with Northern and Western European Ancestry)
CNRQ – log(x) transformed calibrated normalised relative quantification units
CRC – colorectal cancer
CVD – cardiovascular disease
DHF – dihydrofolate
DHFR – dihydrofolate reductase
DHFR-19bp del – DHFR-19 base-pair deletion
DMR – differentially methylated region
DNMT – DNA methyltransferase
dTMP – deoxythymidine monophosphate
dUMP – deoxyuridine monophosphate
HAT – histone acetyltransferases
HCC – hepatocellular carcinoma
HDAC – histone deacetylases
HDL – high density lipoprotein
HMGA – high-mobility group A
Igf2 – insulin-like growth factor 2
INPP5D – phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
KRAS – V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOD – logarithm of odds
LS – least squares
MBP – methyl-CpG-binding proteins
Md – methylation-dependent
miRNA – microRNA/s
mRNA – messenger RNA
Ms – methylation-sensitive
MSP – methyl specific PCR
MTHFR – methylenetetrahydrofolate reductase
MTR – methionine synthase
MTRR – methionine synthase reductase
TOMS – total ozone mapping spectrometer
PBCs- Peripheral blood cells
PCR – polymerase chain reaction
PDCD4 – programmed cell death 4
pre-miRNA – precursor miRNA
pri-miRNA – primary miRNA
PTEN – phosphatase and tensin homolog
qPCR – quantitative PCR
snRNA – small nucleolar RNA
RFLP – restriction fragment length polymorphism
RHLS – retirement health and lifestyle study
RISC – the RNA-induced silencing complex
RXR – retinoic acid receptor
RFLP – restriction fragment length polymorphism
SAH – S-adenosylhomocysteine
SAM – S-adenosylmethionine
SERPINB5 – mammary serine protease inhibitor clade 5
SHMT – serine hydroxymethyltransferase
SOX5 – Sex Determining Region Y-Box 5
STAT3 – signal transducer and activator of transcription 3
THF – tetrahydrofolate
TPM1 – tropomyosin 1
VDR – vitamin D receptor
VDRE – vitamin D response element
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