Urinary Metabolomics of Gastric Cancer

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Clinical Epidemiology

School of Public Health
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Abstract

Gastric cancer is an aggressive malignancy. Much of the mortality is attributable to delayed diagnosis from non-specific symptoms, and lack of early and accurate screening modalities. Metabolomics, the most downstream of the "omics" sciences (genomics, transcriptomics, proteomics) is the latest tool to join the diagnostic armamentarium. The transformation from normalcy to malignancy is accompanied by a series of aberrant biochemical and metabolic alterations. Through detection of metabolites from such pathways, metabolomics may offer potential for early and non-invasive detection of gastric cancer.

Hydrogen nuclear magnetic resonance spectroscopy was used as the analytical platform to explore the urinary metabolomic profile of patients with gastric cancer, in comparison to patients with benign gastric disease and healthy controls who were age, sex and body mass index matched. On multivariate statistical analysis, gastric cancer individuals had a discrete urinary metabolomic signature that was clearly distinguishable from healthy patients, and a subset of benign gastric disease individuals, namely those with chronic gastritis and ulcers. LASSO logistic regression generated a parsimonious model with three metabolites (alanine, 2-hydroxyisobutyrate, 3-indoxylsulfate) that discriminated gastric cancer from healthy controls with high accuracy, sensitivity and specificity. These preliminary results suggest that there is clinical potential for metabolic profiling for gastric cancer detection; however, future studies will be required to validate these findings.

Preface

This thesis is an original work by Angela W. Chan. The research project, of which this thesis is a part, received research ethics approval from University of Alberta Health Research Ethics Board, "Urinary Metabolomics of Gastric Cancer", Pro00037452, August 2013.

Chapter 2 of this thesis has been published as A.W. Chan, R.S. Gill, D. Schiller, M.B. Sawyer, "Potential role of metabolomics in diagnosis and surveillance of gastric cancer," *World Journal of Gastroenterology*, volume 20, issue 36, 12874-12882. AW Chan performed the literature search and wrote the original manuscript. All authors collectively revised the manuscript and prepared it for submission.

Acknowledgements

I would first like to express my sincere thanks to my MSc supervisory committee (Drs. David Broadhurst, Daniel Schiller, Dean Eurich and Michael Sawyer) for their contributions over the last two years. Their support has been key in the completion of this work. I am also grateful to the Clinical Investigator Program at the University of Alberta for this opportunity to conduct dedicated research during my General Surgery residency. Special thanks to Dr. Pascal Mercier for being so kind to run my urine samples at night on multiple occasions; I am very appreciative of his invaluable expertise in profiling spectra with the Chenomx software. Thank you to Rose Cornand, medical secretary for helping me recruit and consent patients, as well as the clinicians and nurses at the Royal Alexandra and University Hospital endoscopy suites for their patience and assistance during the patient enrollment process. Thank you to laboratory technicians, Michelle Kuzma and Delores Mowles, for their assistance in preparation of urine samples for NMR. I am grateful for the multiple sources of funding for this research program (Alberta Innovates Technology Futures Graduate Scholarship, Edmonton Civic Fund, Dr Schiller Academic Enrichment Fund, and Queen Elizabeth II Graduate Scholarship). Lastly, I am grateful to my family (my parents, Bill and Tina Chan, and my siblings, Rocky, Jenn and Lisa Chan) for their long-term support.

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List of Abbreviations

AJCC- American Joint Committee on Cancer

ANOVA- analysis of variance

AuROC- area under receiver operating curve

BMI- body mass index

BN- benign gastric disease

CAG- chronic atrophic gastritis

CDH-1- E-cadherin gene

CSG- chronic superficial gastritis

DSS- 4,4-dimethyl-4-silapentane-1-sulfonic acid internal standard solution

DYS- dysplasia

EBV- Epstein Barr virus

EMR- endoscopic mucosal resection

ESD- endoscopic superficial dissection

EUS- endoscopic ultrasound

FAP- familial adenomatosis polyposis

FDA- Food & Drug Administration

FID- free induction decay

GC- gastric cancer

HDGC- hereditary diffuse gastric cancer

HE- healthy

HMDB- Human Metabolome DataBase

^{1}H-NMR- hydrogen nuclear magnetic resonance spectroscopy

HNPCC- hereditary non-polyposis colorectal cancer

H. pylori- Helicobacter pylori

IM- intestinal metaplasia

MALT- Mucosal associated lymphoid tissue

MS- mass spectrometry

MVA- multivariate analysis

OPLS-DA- orthogonal partial least squares-discriminant analysis

PC- principal component(s)

PCA- principal component analysis

PG- pepsinogen

PLS-DA- partial least squares- discriminant analysis

PPI- proton pump inhibitor

ppm- parts per million

PQN- probabilistic quotient normalization

Q^2- goodness of prediction statistic

QC- quality control

R^2- goodness of fit statistic

ROC- receiver operating characteristic

RSD- relative standard deviation

SOP- standard operating procedure

TNM- Tumour node metastasis staging system

TMS- tetramethylsilane internal standard solution

UV- unit variance scaling

US- United States

CHAPTER 1: INTRODUCTION

1.1 Overview of Gastric Cancer

1.1.1 Gastric cancer epidemiology

Gastric cancer (GC) is the fifth most common cancer worldwide[1] and the third most deadly.[2] It represents about 7% of all cancers, after lung, breast, colorectal and prostate respectively. About one million people are diagnosed worldwide every year,[1] and there is a 70% mortality rate.[2] Premature death and disability from GC has a large economic impact. In 2008, the American Cancer Society estimated that countries ranked in the second lowest quartile by per capita gross domestic product, lost 4.8 million disability adjusted life years (DALY) in one year due to GC. This translates into 10.2 billion US dollars lost per year due to death/disability.[3]

There are geographical differences in GC distribution. Sixty percent of cases occur in East Asia, with Korea, Mongolia and Japan having the top three prevalence rates.[4] GC is considerably less common in Canada; in 2013, 3300 cases were diagnosed nationwide and GC contributed to 2-3% of all cancer related deaths.[5] Males are more commonly affected than females, with a 2:1 incidence ratio.[6] The average age of diagnosis is 69 years old.[7]

1.1.2 Gastric cancer histology

The most common histological subtype of GC is adenocarcinoma, which comprises 95% of GCs. The Lauren classification divides adenocarcinomas into diffuse or intestinal subtypes. The intestinal type is found more commonly in high incidence geographic regions and is characterized pathologically by the tendency of malignant cells to form glands. They generally spread hematogenously and are more frequent in older patients; most of the time these tumours are well to moderately differentiated. On the other hand, the diffuse type lacks organized glands, has many signet ring cells, and is poorly differentiated. Signet ring cells occur when greater than half the tumour contains intracytoplasmic mucin. Diffuse type tumours commonly afflict younger and more obese patients. They can spread via lymphatics and transmurally.[8]

Since the 1980s, the incidence of proximal gastric tumours (upper one-third of stomach) is rising, especially in North America. Proximal tumours are associated with worse outcomes.[9] Cancers of the gastric cardia account for nearly half of all adenocarcinomas. Just under 10% of all tumours involve the entire stomach; this is known as *linitis plastica* or leather bottle stomach and it carries a dismal prognosis. Commonly gastric cancers metastasize to the liver, lungs and peritoneum.[10]

1.1.3 Risk Factors

There are some well-established risk factors for GC. Genetic, inflammatory, dietary, lifestyle, infectious and ethnic factors contribute to the multifactorial pathogenesis of GC. High intake of salt has long been associated with increased GC

morbidity and mortality. In one prospective cohort study, subjects with a dietary intake of salt ≥10 g/day had an age and sex adjusted hazards ratio 2 times higher than subjects with a dietary intake <10 g/day.[11] The authors postulated that excess salt alters the viscosity of the mucus lining. Once this mucosal barrier is weakened, carcinogenic agents such as nitrates can come into contact with the gastric mucosa. Alternatively, high salt intake can facilitate colonization by *Helicobacter pylori (H. pylori)* bacteria. There may be a synergistic connection between salt and bacteria. With increased refrigeration, the frequency of salt use as a preservative has declined, as have GC mortality rates.[12] Fruits and vegetables are a protective factor; in one prospective study, there was a 44% reduction in GC associated with 2-5 daily servings compared to 1 serving. Smoking and tobacco use was correlated with a 1.53 increase in relative risk of developing GC, and the risk was dose dependent.[12]

A body mass index (BMI) of 25-30 kg/m^2 is associated with a 1.71 fold increased risk of GC, whereas a BMI over 30 kg/m^2 confers a 2.34 fold relative risk compared to normal BMI.[13] Ethnic groups at higher risk for GC include East Asians and First Nations who also have high rates of *H. pylori* infection and seropositivity.[14] Another infectious agent implicated in GC is Epstein-Barr Virus (EBV) which occurs in 10% of GC cases.[6] Other miscellaneous factors associated with gastric carcinogenesis include pernicious anemia (an autoimmune disorder where gastric parietal cells are destroyed), prior gastric surgery for benign disorders such as ulcers, and ionizing radiation.[6]

About 3-5% of GCs have a genetic predisposition.[15] E-cadherin (CDH1) mutations are found exclusively in the diffuse type of GC, whereas no genetic

associations have been identified yet in the intestinal type of GC. People with CDH1 mutations have as high as an 80% lifetime risk of developing hereditary diffuse gastric cancer (HDGC). This tumour is known for multiple areas of signet ring carcinoma initially confined to the superficial lamina propria in the mucosa.[16] However most cases of HDGC eventually spread and cause death. GC may also appear alongside other hereditary cancer syndromes such as hereditary non-polyposis colon cancer (HNPCC), Li-Fraumeni syndrome, familial adenomatosis polyposis syndrome (FAP), and Peutz-Jeghers' syndrome.

1.1.4 Helicobacter pylori infection

Helicobacter pylori infection is the most significant risk factor for distal (non-cardia) gastric cancer. *H. pylori* is a Gram negative bacterium that colonizes the distal stomach. In 1994, the International Agency for Research on Cancer classified this bacterium as a Class I carcinogen,[17] as it was frequently associated with GC and mucosal associated lymphoid tissue lymphoma (MALT). It also plays a role in peptic ulcer disease and chronic gastritis. About 50% of the world's population is infected; however, most are asymptomatic. Infection rates are highest in developing countries, and it is tied to lower socioeconomic status, more specifically overcrowding and sanitation. The odds of developing GC in patients infected with *H. pylori* are about three times greater than those who are not infected.[6]

H. pylori inhabits the mucus lining of the stomach. It produces urease, which converts urea to carbon dioxide and ammonia. The ammonia protects *H. pylori* from the harsh acidic environment in the stomach lumen. *H. pylori* induces

inflammation and damage to the epithelial cells, resulting in a chronic gastritis. A recent basic science study demonstrated that *H. pylori* induced double stranded breaks in DNA in gastric cell lines *in vitro*, setting the stage for carcinogenic mutations.[18]

H. pylori inflammation leads to chronic atrophic gastritis (CAG), which was implicated long ago to be an early step in the carcinogenesis pathway. In 1988, Peyo Correa published an article studying GC progression in mice. The progression sequence is from chronic superficial gastritis (CSG) to CAG to intestinal metaplasia (IM) to dysplasia (DYS) to intestinal GC.[19] As inflammation progresses, there is loss of pepsinogen I and II, two pro-digestive enzymes produced predominantly in chief cells of the stomach. Loss of chief cells decreases serum levels of pepsinogen I. Pepsinogen I levels below 20 ng/ml are closely correlated with atrophic gastritis. On the other hand, diffuse type GC does not progress through severe atrophic gastritis.[20]

Chronic gastritis induced by *H. pylori* is the strongest known risk factor for GC. Development of subsequent GC seems to hinge greatly on persistence of *H. pylori* infection. An animal study by Romero-Gallo demonstrated that Mongolian gerbils infected with *H. pylori* all developed gastritis; however, those that were treated appropriately with antibiotics resulted in attenuation of pre-malignant and malignant phenotypes compared with controls who were not given antibiotics.[21]

There are multiple ways to test for *H. pylori*. Non-invasive techniques include the radioactive carbon urease breath test, stool antigen test, and blood IgG antibody test. Endoscopic techniques include forceps and brush biopsy. Each test

has its own sensitivity and specificity. Eradication of *H. pylori* can be achieved with antibiotics; the aforementioned tests can be used to confirm effective treatment. Increased use of *H. pylori* antibiotics is correlated with a decreased incidence and mortality of gastric cancer.[6]

1.1.5 Screening methods

Stomach cancer is often diagnosed late as signs and symptoms do not appear until the malignancy is reasonably advanced. On average, it takes about 44 months for GC to progress to an advanced stage.[22] Commonly patients present with vague epigastric pain, weight loss, anemia of unknown origin, and upper gastrointestinal bleed. Stomach cancers that do not penetrate beyond the mucosa are often asymptomatic. The delay in diagnosis can also be attributed to the widespread use of proton pump inhibitors (PPI). Dyspeptic symptoms of early GC are similar to that of ulcer disease, and as such, can be passed off as benign. PPIs mask GC symptoms leading to delayed recognition of malignancy. There is a possibility that when patients undergo endoscopy, early gastric cancers may have healed after a short course of PPI. For these reasons, it is recommended that patients over age 45 years with new onset of dyspepsia be referred first for endoscopy prior to receiving PPI therapy.[23]

There is currently no population based screening program in Canada or the United States, as the prevalence of GC is very low. However, in countries such as Japan and Korea where GC prevalence is high, screening has been cost effective. Barium photofluorography, endoscopy and serum pepsinogen testing are common

modalities to detect early GC.[24] The limitations of endoscopy are that it is operator dependent and not available in all centers.

Serum pepsinogen (PG) is another diagnostic marker of atrophic gastritis and possibly GC. Pepsinogen I is a proenzyme produced almost exclusively by chief and mucous neck cells in the fundic glands, while Pepsinogen II is produced by chief cells and also by pyloric glands/Brunner glands. Serum and tissue concentrations of PG I and PG I/II ratio showed a progressive decline in the sequence of conditions outlined by Correa (normal to CSG to CAG to GC).[25] A recent Korean study defined gastric atrophy as PG I level <70 ng/mL and a PG I/II ratio <3. These values had sensitivity of 77%, a false positive rate of 27%, and a high negative predictive value of >99%.[26] In both Japan and Korea, these are the accepted PG cut-off values for GC detection. Serum pepsinogen can also be affected by other factors including age, gender, BMI, body surface area, smoking and diet.

Table 1-1: Sensitivity and specificity of GC screening modalities

Modality	Sensitivity	Specificity
Barium photofluorography[27]	60-80%	80-90%
Conventional endoscopy[28]	69%	96.0%
Endoscopic ultrasound[24]	T staging- 86%	T staging- 91%
Serum pepsinogen[29] For PG I ≤70 & PG I/II ≤3.0	77%	73%

1.1.6 Staging and Prognosis

Canada does not have its own staging system for stomach cancer, but it does follow the American Joint Committee on Cancer (AJCC) guidelines. GC can be classified into four stages based on various combinations of TNM

(tumour/node/metastasis) statuses. Tables 1-2 and 1-3 show the most recent (7th edition) classification system.[30]

Commonly employed staging modalities include EUS, computed tomography (CT) of the chest/ abdomen/pelvis, and staging laparoscopy. EUS is most useful in determining depth of invasion (T stage), especially distinguishing between mucosal (T1a) versus submucosal (T1b) lesions. Only T1a cancers are amenable to endoscopic mucosal resection.[31] CT, on the other hand, is useful for evaluating distant metastases in the chest and abdomen. However, early small malignant lesions (<1 cm diameter) can be missed on the CT scan, which is where staging laparoscopy becomes a useful adjunct. The four quadrants of the abdomen can be visualized with a camera and any suspicious nodules biopsied. Commonly peritoneal lavage with normal saline is also performed for tumour cytology. With diagnostic laparoscopy, approximately one-quarter of patients can be spared a laparotomy if peritoneal metastases are identified.[32] In this way, staging laparoscopy provides additional information about which patients have resectable disease.

Table 1-2: TNM classification of gastric cancer

T category	N category	M category
Tx = primary tumour not able to be assessed	Nx = regional lymph nodes cannot be assessed	M0 = no spread to distant organs
Tis = tumour in situ	N0 = no regional lymph node metastasis	M1 = spread to distant organs
T0= no evidence of primary tumour	N1 = 1-2 regional lymph nodes involved	
T1= tumour invades submucosa	N2 = 3-6 regional lymph nodes involved	
T2 = tumour invades muscularis propria	N3a = 7-15 or more regional lymph nodes involved	
T3 = tumour invades subserosal connective tissue without invasion of serosa. May have spread into gastrocolic, gastrohepatic ligaments, greater or lesser omentum, no perforation of serosa	N3b = ≥16 lymph nodes involved	
T4a= tumour invades serosa		
T4b = tumour invades adjacent structures such as spleen, colon, liver, etc.		

Table 1-3: GC Staging based on TNM category combinations

Stage	T category	N category	M category
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
	T1	N1	M0
IIA	T3	N0	M0
	T2	N1	M0
	T1	N2	M0
IIB	T4a	N0	M0
	T3	N1	M0
	T2	N2	M0
	T1	N3	M0
IIIA	T4a	N1	M0
	T3	N2	M0
	T2	N3	M0
IIIB	T4b	N0 or N1	M0
	T4a	N2	M0
	T3	N3	M0
IIIC	T4b	N2 or N3	M0
	T4a	N3	M0
IV	Any T	Any N	M1

Due to late diagnosis of stomach cancer, the prognosis of patients is generally poor. Table 1-4 summarizes the 5-year survival for various stages of GC.[30, 33]

Table 1-4: Five-year survival rate of GC patients by stage

Stage	**5-year survival rate (%)**
IA	71
IB	57
IIA	46
IIB	33
IIIA	20
IIIB	14
IIIC	9
IV	4

After achieving an R0 resection (complete removal of tumour with margins microscopically negative for cancer), the strongest prognostic factor is lymph node involvement. Other prognostic factors that affect overall survival are: tumour size (<3 cm), depth of invasion (superficial tumour infiltration), tumour differentiation (lower grade tumours), and Lauren histological class (intestinal type cancers).[34] Favourable factors are indicated in brackets.

1.1.7 Treatment modalities

As with most other cancers, GC therapy consists of a three-pronged approach: chemotherapy, radiotherapy and surgery. After all staging investigations are completed, patients are divided into either resectable or non-resectable disease groups. Early gastric lesions defined as being confined to the mucosa or submucosa of the stomach, <2 cm and non-ulcerated can be managed endoscopically either with endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD). These advanced techniques are more commonly practiced in East Asia, but are spreading to the west.

For lesions that have extended beyond submucosa but are not yet metastatic, either a subtotal or total gastrectomy is performed with lymphadenectomy. The goal is for complete removal of tumour with a 5 cm proximal margin, as well as a minimum of fifteen lymph nodes. A lymphadenectomy can be D1 (perigastric lymph nodes only), D2 (D1 + celiac trunk lymph nodes) or D3 (D2 + periaortic lymph nodes). A large multi-institutional Dutch trial randomized over 700 resectable GC

patients to either a D1 or D2 lymphadenectomy. Follow-up at fifteen years demonstrated that D2 lymphadenectomy was associated with lower locoregional recurrence rates and GC related mortality.[35] However, overall survival was no different and in fact, the morbidity and mortality of the D2 group was significantly higher than the D1 group. Similar results were found in the British Medical Research Council Gastric Cancer trial.[36] While surgery is the mainstay of treatment, GC tends to be very aggressive, and often neoadjuvant and adjuvant chemoradiotherapy are needed to boost cure rates.[37, 38] Usually a multidisciplinary tumour board decides on a patient's candidacy for non-surgical therapies.

1.1.8 Cancer and Metabolic Dysregulation

Altered metabolism is one of the hallmarks of cancer. Malignant cells show enhanced ability to ferment glucose into lactate, even in an environment where there is sufficient oxygen to support mitochondrial metabolism. This phenomenon is known as the Warburg Effect.[39] Multiple in-vitro studies show elevated lactate levels in GC cells compared to healthy controls.[40-42] Cancer cells also express high levels of glutamine, an amino acid, which acts as a substrate for lipogenesis and nucleic acid synthesis. These processes are important for cell membrane synthesis and DNA replication. Other features of tumours include the ability to evade regular apoptotic checks and balances. In GC cell lines in particular, citrate (an intermediate of the Kreb's cycle) has been implicated in regulating apoptosis.[43] It is apparent that a number of metabolic pathways are affected during GC tumorigenesis and

propagation, so studying the metabolites of such perturbed pathways may offer insight into new diagnostic and therapeutic targets of GC.

1.2 Overview of Metabolomics

Metabolomics is the study of metabolites, which are low molecular weight organic and inorganic chemicals (<1500 Da) in a biological organism. Metabolites can be reactants, intermediates or products of any enzyme mediated reaction. Metabolites are formed from anabolic and catabolic reactions, and can be influenced by environmental factors such as drugs and diet.[44] Metabolomics is the latest addition to the "omics" family of systems biology. According to the central dogma, genes are transcribed into mRNA, which are translated into proteins and finally broken down into metabolites. Mirroring this flow of biological information is the study of genomics (genes), transcriptomics (mRNA), proteomics (proteins) and metabolomics (metabolites) respectively. This flow is illustrated in Figure 1-1. Metabolomics, the most downstream of the "omics" sciences, is closest to the phenotype of an organism. There is bidirectional transfer of information between each functional level, and environmental inputs such as diet, lifestyle, and drugs integrate to create the final phenotype for an organism.

Endogenous metabolites are synthesized by enzymes intrinsic to the body; exogenous metabolites are imported from outside sources (drugs, diet). Metabolites can also be classified as primary or secondary. Primary metabolites are essential for growth, maintenance and reproduction of an organism. The main classes of

endogenous metabolites are: lipids, alcohols, vitamins, carbohydrates, organic acids, nucleotides, and amino acids. Secondary metabolites are not required for survival but may still have an important ecological function.[44, 45] Mapping the metabolomic profile provides a global picture of the organism at a specific point in time under a specific set of conditions. For any given disease state, a small genomic change can be amplified many times at the metabolite level and quantified. The human metabolome consists of thousands of metabolites, many of which are listed in the *Human Metabolome Database (HMDB).*[46] HMDB contains comprehensive descriptions of individual metabolites and can be accessed online. As of 2013, over 40,000 metabolites are in the HMDB library.[47]

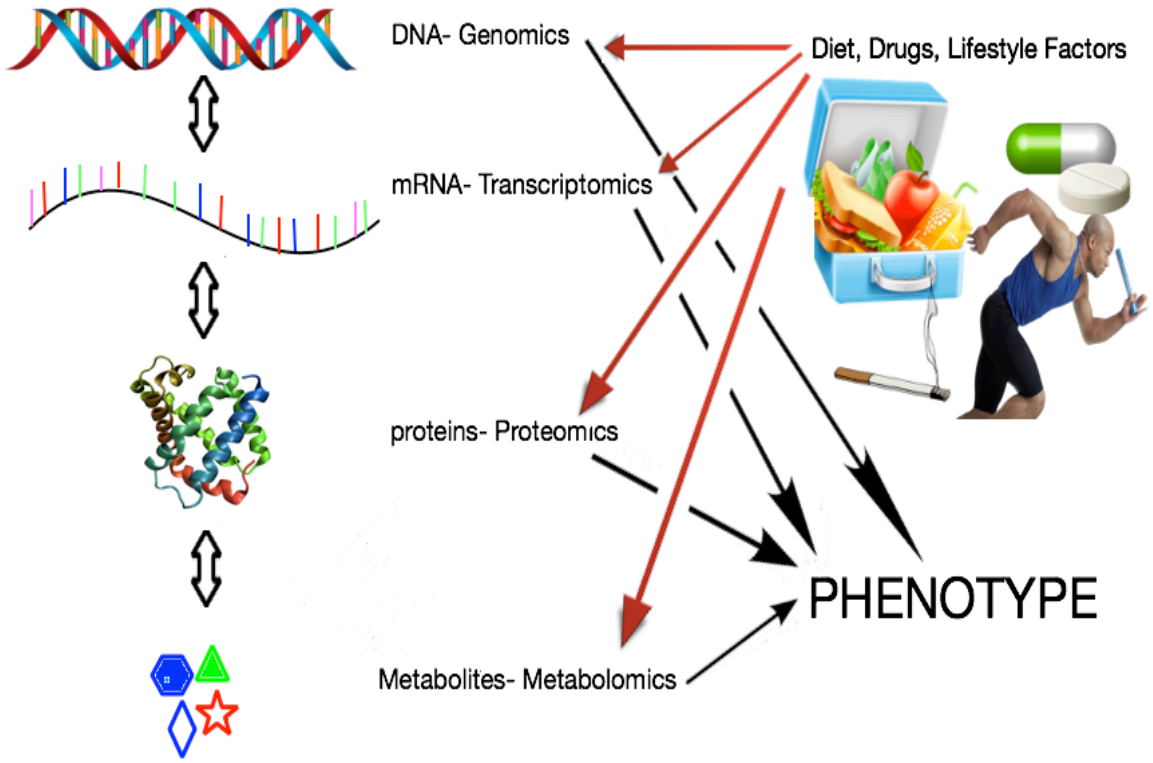

Figure 1-1: Central Dogma of Biology
Biological information flows from genes (genomics) to metabolites (metabolomics) to create the final phenotype. There is bidirectional crosstalk between each step of the pathway as well as influence from drugs, diet and lifestyle.

Metabolites can be extracted, identified and quantified using human tissues, and biofluids such as urine, blood, and cerebrospinal fluid. Biofluids are easily collected via minimally invasively techniques, whereas human tissues generally require lysis and homogenization to extract metabolites. Validated standardized operating procedures for collection, storage, and processing are employed to ensure each sample is subjected to the same procedures. Metabolites are very sensitive to enzymatic and environmental conditions such as temperature and pH. There are two key steps in sample collection and preparation: a) "quenching" of metabolic activity and b) extraction of metabolites into an appropriate medium for analysis.[44]

Quenching is a process where metabolic activity is stopped to capture an instant snapshot of metabolism at a certain time under a given set of conditions. Decreasing the temperature of the sample is a common way to inactivate enzymes. Samples are usually collected and stored in -80° Celsius (C) as soon as possible. This temperature is usually enough to stop enzymatic activity, although after a few years, there is still the possibility of metabolic change. Once metabolites are extracted from the tissue or biofluid, they often need to be solubilized into an organic solvent such as ethanol or methanol.[44]

1.2.1 Analytical Platforms

There are a number of analytical platforms for metabolomics, including mass spectrometry (MS), ^{1}Hydrogen- nuclear magnetic resonance (^{1}H -NMR) spectroscopy, gas and liquid chromatography. Often several techniques are combined to improve metabolite identification. In chromatography, the sample, known as the mobile phase, exists in either gas or liquid form. It is held within a glass or metal column. The mobile phase is forced through a stationary phase held in a column or solid surface. The stationary phase is usually an inert substance that does not react with the mobile phase. Some components of the solute will be adsorbed onto the stationary phase, while other components will still be mobile; it depends on their chemical properties. Volatile materials migrate through the column more rapidly.[48] A recording device generates a series of peaks, which show the overall retention time of the compound in the chromatograph.

The two most common analytical platforms are MS and NMR. As previously mentioned, MS separates ions in metabolites based on their mass-to-charge ratio, whereas NMR separates metabolites by their resonance frequencies in a magnetic field. Both techniques generate a spectral profile of metabolites. Both NMR and MS can identify and quantify a wide variety of metabolites with good precision, and both require small sample volumes for analysis (10-700 uL).[49] NMR is highly reproducible and does not rely on component separation so samples can be recovered for future analysis.

Sensitivity of an analytical method refers to the minimum amount of sample compound that is required for quantification.[50] Instruments that are more sensitive can detect metabolites that are present in smaller quantities. For comparison, MS can typically detect metabolites present in nanomolar or picomolar concentrations, whereas the limit of detection for NMR is on the order of micromolar concentrations.[51] NMR is also less resolved than MS because of co-resonant metabolites (overlapping peaks in the same region of the NMR spectrum), which can limit accurate detection in that particular region.[44, 50] As NMR is the platform of choice for experiments in this thesis, its basic physics principles will be reviewed here.

1.2.2 ^{1}Hydrogen (^{1}H)-NMR Basic Principles

Any nucleus with an odd atomic number such as ^{1}H possesses spin, which generates its own magnetic field. In ^{1}H-NMR, samples are placed inside an apparatus with a large external magnetic field.[52] Magnetic moments of nuclei in the

sample either align with the external magnetic field (lower energy conformation) or against the magnetic field (higher energy conformation). If the sample is irradiated with radiowaves, protons (^1H) in the sample absorb the electromagnetic radiation and spin flip to a higher energy state;[53] this process of energy absorption is known as resonance. When protons fall back to ground state, they emit a radiofrequency signal that is directly proportional to the strength of the external magnetic field. A radiofrequency receiver on a computer captures this, and translates the signal into a series of peaks with a characteristic chemical shift (Figure 1-2).

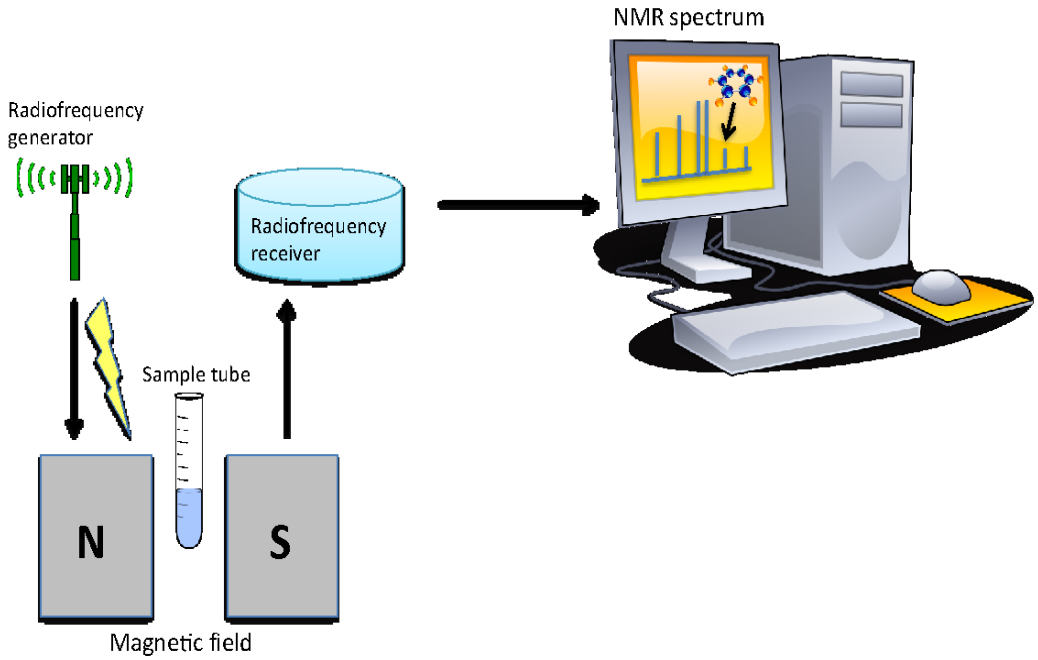

Figure 1-2: Schematic of NMR spectrometer.
Radiowaves irradiate the sample placed inside a magnetic field inside the NMR machine. A radiofrequency receiver detects the energy released by the protons in the sample, and transmits this information to an NMR program, which produces a spectrum.

As nuclei lose energy, the radiofrequency signal gradually diminishes in a free induction decay (FID) pattern; the FID contains the sum of the frequencies from all nuclei and is visualized as a voltage versus time plot. A single FID has a low

signal to noise ratio, but after repeated acquisitions, this ratio improves proportionally with the square root of the number of acquisitions.[53] To double the signal to noise ratio, the FID signal must be acquired four times. A mathematical function known as a Fourier transformation converts a time domain FID into a frequency domain spectrum.[52, 53] This results in a series of peaks with a certain chemical shift. The area under the peak is proportional to the relative concentration of the metabolite.

Prior to operation of the NMR magnet, a procedure called shimming is used to make the magnetic field more homogenous. Shimming improves the sensitivity and resolution of the acquired signals.[54] Each biofluid sample must be mixed with an internal standard in order to generate a peak on the spectrum. Tetramethylsilane (TMS) or 2,2-dimethyl-2-silapentane-5-sulfonate-d6 acid (DSS-d6) are common standards for ^{1}H-NMR spectroscopy.[55] The internal standard is set to a reference chemical shift of 0 ppm; left of 0 ppm are increasing chemical shifts in the direction of increasing resonance frequency. A chemical shift is defined as the difference in parts per million (ppm) between the resonance frequency of the observed proton in the sample and the internal standard, divided by the spectrophotometer frequency.[52] The chemical shift is also known as the delta (δ) scale.

1.2.3 Metabolite profiling strategies

Profiling of metabolites can take an untargeted, targeted, or semi-targeted approach. Untargeted profiling involves acquiring data on hundreds to thousands of

metabolite features without a priori knowledge of biologically relevant metabolites. There is no definitive identification and quantification. On the other hand, targeted profiling quantifies a smaller number of known metabolites (typically fewer than 20), in an attempt to create systems biology models. Targeted profiling is useful for hypothesis testing.[44, 49] Semi-targeted analysis identifies and measures pre-defined metabolites of interest.[49] Usually these metabolites are referenced from a library.[56] Both untargeted and semi-targeted techniques are for hypothesis generation. The choice of metabolic profiling approach is determined by the hypothesis of the study. In this thesis, a semi-targeted method is employed, as metabolites are identified and quantified from a known reference library (Chenomx).

1.2.4 Metabolomic Workflow

Once the biofluid or tissue has been chosen, metabolites extracted and isolated for analysis, then metabolomics studies usually follow an experimental workflow known as the "metabolomic pipeline".[44] Steps are delineated in Figure 1-3.[44, 49, 57] It starts with proper experimental design, instrument (analytical platform) setup, analysis, and then capturing of raw data. Once data is acquired, then it undergoes pre-processing to generate a "cleaned" data set, which is then analyzed through a number of statistical techniques. Components of this workflow will be discussed in greater detail later.

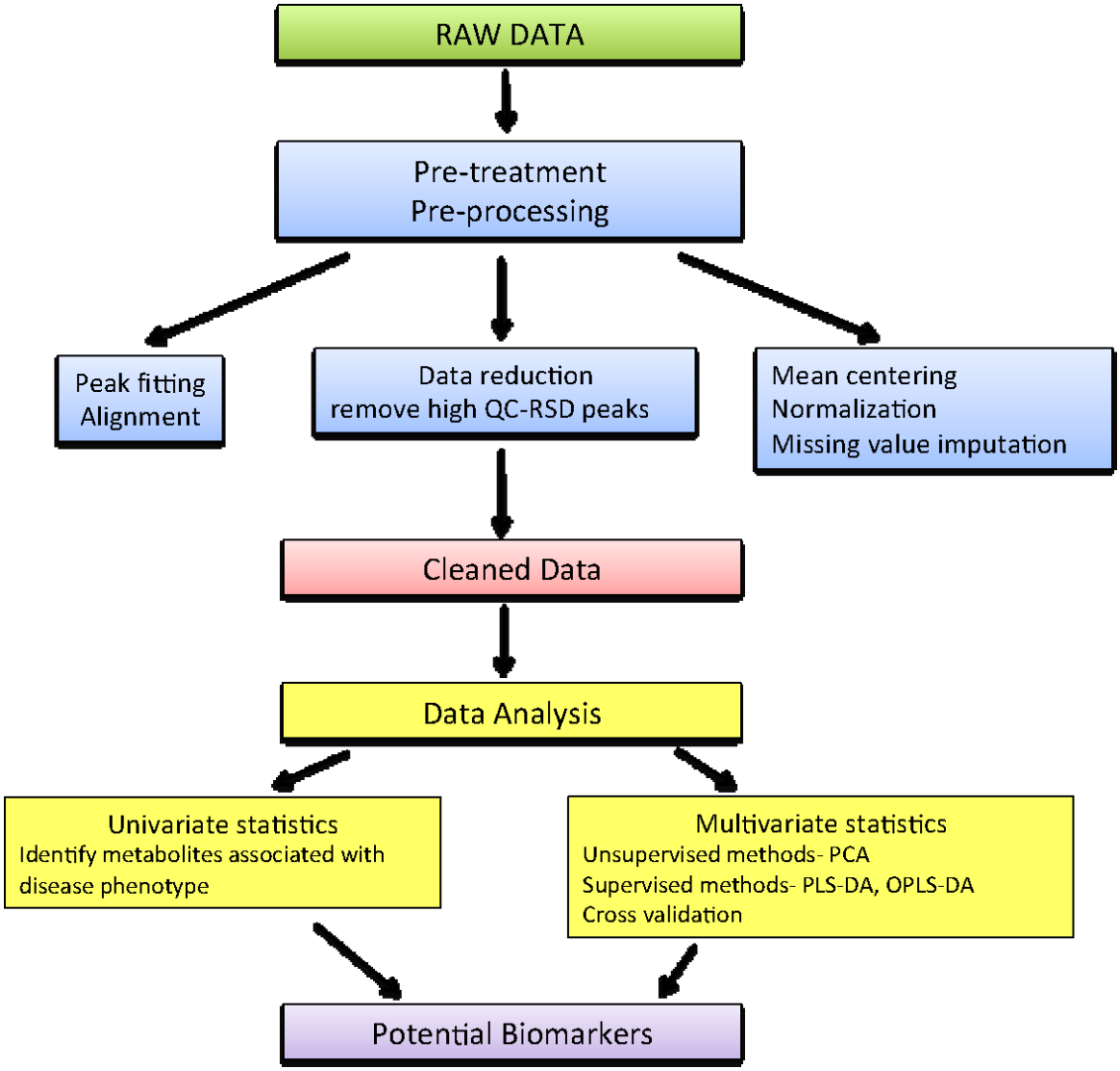

Figure 1-3: The Metabolomic Pipeline. This flowchart illustrates the steps required to convert raw data into a list of potential biomarkers.

1.2.5 Experimental Design

A valid and robust scientific study begins with a proper study design. Every effort is made to ensure that variation related to biological observations is significantly greater than the variation related to performing the study.[56] Bias and confounding can be introduced in either or both of the design and analysis stages of the experiment and this can lead to false observations and biological conclusions.

Goodacre *et al*[58] described two important stages of experimental design. First there is the biological study itself, which involves the rigorous collection, storage, and processing of study samples. There should be a validated standardized operating procedure (SOP) such that different people collecting samples on different days at different centers will perform the same procedure. This reproducibility helps minimize selection bias. Data related to demographics, lifestyle and physiological variables such as diet, gender, ethnicity, and BMI should be collected where possible, as these may be confounding factors later. As well, sample size should be determined prior to sample collection, to ensure that a study is sufficiently powered to detect a difference.

The second component is the analytical design. In human studies, patients enrolled are generally not from a random sample, but rather a convenience sample. Subjects attend a collection site such as a hospital, or outpatient health clinic and are approached if they meet the inclusion/exclusion criteria. While sampling methods are not randomized, researchers can randomize both sample preparation and analysis order. It is best if patient identities are blinded to the researcher during experimentation and analysis to reduce bias. Patients are enrolled by a convenience sampling method, but their run order in the experiment can be randomized. Run order refers to the order in which patient biofluid or tissue samples are placed through the detection device (NMR or MS, or other platform). Often, in large studies, all samples cannot be run on the same day. This necessitates splitting of samples into smaller analytical experiments, or batches. With successful

randomization, the distribution of subjects across each batch should be more or less similar.[56]

For quality assurance purposes in metabolomics experiments, a quality control (QC) sample should be analyzed intermittently. A QC is a biologically identical aliquot of either one person's biofluid or tissue sample, or a pooled mixture of multiple patients. For example, 50 uL aliquots each of five different patient's urine samples can be combined to make one QC. The same SOP that was used to collect, store and process study samples should be applied to QCs. Variation in the QCs represents the overall within-experiment precision. Error can be introduced in the experimenter techniques and measurement devices, which can be captured in the QC variation.[56]

To quantify variation in the QC in metabolomics experiments, the relative standard deviation (RSD) can be calculated by dividing the standard deviation for each metabolite by the mean concentration of each metabolite. The Food and Drug Administration (FDA) guidelines recommend that for single analyte tests, the QC-RSD should be ≤15%, except for metabolites whose concentrations are at or near the lower limit of quantification, in which case a QC-RSD of ≤20% is acceptable.[44] The FDA recommends QC-RSD ≤20% for ultra performance liquid chromatography-mass spectrometry (UPLC-MS) and QC-RSD ≤30% for gas chromatography-MS. Any metabolites for which the QC-RSD exceeds industry standards are not considered in the analysis. Only metabolite peaks in which ≥50% of the QCs express the same peak are preserved for analysis. The less variable the QCs are, the more confidence

the researcher has that observed differences between samples are biologically significant.[56]

1.2.6 Data Pre-processing

After a rigorously designed experiment is completed, raw spectroscopic data must be pre-processed. There are multiple steps of pre-processing as outlined by Goodacre and colleagues.[58] For NMR data, raw FID weighting, phasing, baseline correction with referencing to an internal standard, normalizing to spectral area and conversion to magnitude spectra are components of pre-processing. Then data undergoes pre-treatment which consists of determining bin sizes of chemical shifts (if a binned analysis is utilized in NMR) and integrating intensities in chemical shifts. Pre-treatment involves other mathematical functions such as normalization, mean-centering, scaling, missing value imputation and transformations that make the data more suitable for processing.[58]

Metabolites exist in a given system with a wide range of concentrations. Logically, high concentration metabolites will often have high variance in a given sample, whereas low concentration metabolites often have a low sample variance. As such in order to equalize the "importance" of each metabolite in an unbiased statistical model each metabolite is usually scaled to unit variance (each metabolite is divided by its standard deviation).[59]

After unit variance (UV) scaling comes mean centering. The average value of each variable column (metabolite concentration) is calculated and then the average is subtracted from each row in that variable column. This repositions the

coordinate system, such that the average point is now defined as "zero" (the reference point).[59] Mean centering adjusts for differences between very high and very low abundance metabolites (outlier concentrations); mean centering focuses on the variance not the absolute concentration.[60]

One of the early crucial steps in pre-treatment is sample normalization; there are different techniques: integral normalization, creatinine normalization, probabilistic quotient normalization (PQN).[61] A normalization step prior to data processing compensates for differences in overall concentration amongst samples. A mathematical transformation may also be applied to make skewed data more normally distributed. Standard practice involves a logarithmic or power transformation of data point(s).[60]

1.2.7 Data Analysis: Statistical Methods

Once the data is "clean" then it is suitable for either univariate or multivariate data analysis. Univariate statistics (such as t-tests/Kruskal-Wallis tests)[58] can be used to check whether individual metabolites are significantly increased or decreased between different groups. A single metabolomics experiment can measure hundreds to thousands of metabolites; this can be likened to performing many individual tests simultaneously. As the number of evaluations in an experiment increases, so does the chance of finding a spurious association. Post-hoc statistical tests such as Bonferroni correction and Benjamini-Hochberg False Discovery Rate can compensate for multiple comparisons.[62]

Multivariate statistics analysis can be unsupervised or supervised. Unsupervised methods are used when the information about outcome, or class, is unavailable or not of primary interest. These methods look for naturally occurring clustering of data based on multivariate covariance. The most popular unsupervised method is Principal Component Analysis (PCA). Conversely, supervised methods use the known class membership to guide the clustering process. The objective is to build a linear multivariate model that will correlate as much of the observed data with the class data. This can be considered similar to classical hypothesis testing – such as t-test or ANOVA.

PCA is essentially a dimension reduction technique that summarizes the variance between observed multivariate data points into a few principal components (PC). The first PC explains a certain amount of variation in the data (for example 35%), and the second PC, which is orthogonal to the first, accounts for another portion of the variation in the data (for example 20%). Finding the first PC involves finding an axis in multidimensional space for which the variance is maximized between data points. PCA strives to compress the data while preserving as much of the original information as possible; it therefore provides a global overview of data points that can highlight similarities and differences.[58, 59]

Two popular supervised analytical methods are Partial Least Squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA).[58, 59, 63] Both PLS methods use information about class or outcome to summarize (project) the observed data in terms of discrimination rather than variance. Like PCA, both PLS and OPLS are dimension reduction strategies and

can be expressed in terms of components. The first PLS component strives to find an axis that well approximates the relationship between observed and outcome data points. OPLS-DA is an extension of PLS-DA. It splits the variability (sum of squares) in the observed data into a component that is correlated/predictive of outcome/class, and an orthogonal component that is not correlated/predictive of outcome/class. The correlated part is known as the signal; the uncorrelated part is the noise. This should theoretically improve the interpretability of the model.

In summary, PCA maximizes the *variance* between observations without knowledge of class or outcome variable. PLS –DA models the maximum *covariance* between observed and outcome variables. Cross validation is the method used by PLS to determine the optimal number components that are significant in the model.[59, 64] OPLS-DA is similar to PLS-DA but it subdivides the observed components into a part that is correlated and a part that is not correlated with outcome, to improve biological interpretability.

1.2.8 Model Diagnostics and Validation

In order to determine the appropriate number of PLS components, a cross validation mathematical technique is employed. The optimal PLS model accurately explains a large amount of variance in the model, but also does not "overfit". The goodness of fit can be quantitatively represented by the R^2 parameter. R^2 describes the percent of variance in the data set that is explained by the model. The goodness of prediction from the model is represented by the Q^2 parameter and identified by cross validation. The R^2 and Q^2 parameters generally refer to the outcome data

rather than the observed data. As more variables are added to the model, the R^2 value generally approaches 1, or close to 100% of the variance in the outcome variable can be explained by the observed data. However the Q^2 will plateau at a certain point, regardless of how many additional variables are incorporated into the model. Q^2 values only increase when significant variables are added to the model; Q^2 decreases when "noisy variables" are added. A good model essentially explains as much of the true variation between observed and outcome variables as possible, without describing the noise (random error). At the same time, the relationship being described can also be generalized to a new data set (external validity). An "overfit" model can be likened to connecting the dots on a scatterplot; it perfectly models the relationship between points, but the same relationship cannot be applied to a new data set. On the other hand, an appropriately fit model has a line of best fit that approximates the sample data well, but at the same time, the mathematical relationship being described can be generalized to a new data set.

The optimal number of PLS components occurs in the zone where Q^2 and R^2 are maximized and the difference between the two parameters is reasonably small (Figure 1-3). Some general rules of thumb for quality measurements are that a $Q^2 > 0.5$ is good and $Q^2 > 0.9$ is excellent in its predictive ability (although these numbers are application dependent). Differences between R^2 and Q^2 should not be larger than 0.2.[59]

To determine the optimal number of PLS components, cross validation is performed by dividing the data into a number of groups, and then excluding one group systematically to produce a number of independent parallel models from a

subset of data. The excluded set is called the hold out, and the remainder of the data that is used to build a model is called the training set. In some software programs such as SIMCA (Umetrics, Sweden), the default cross validation method divides the dataset into seven groups. On each round of validation, six-sevenths of the total data is used to generate a model prediction. Models built using the training data can then be independently validated with the omitted set. After seven rounds of validation, seven models with seven individual Q^2 values are generated; the Q^2 values are averaged together to create the final Q^2 in the overall PLS model.[44]

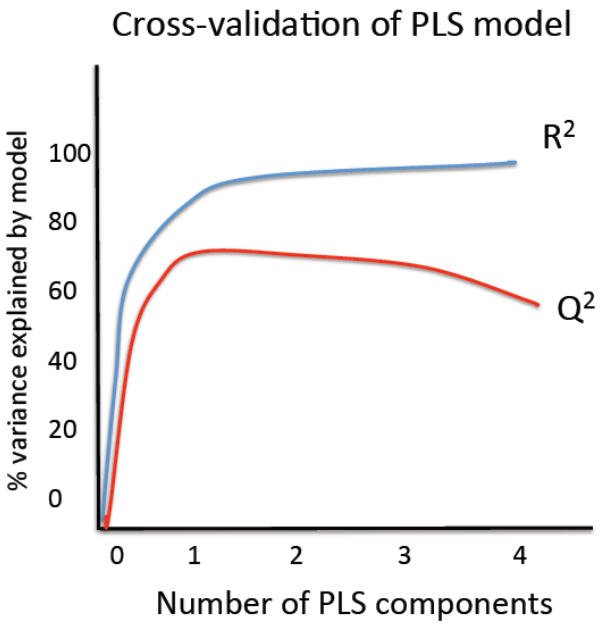

Figure 1-4: Cross validation model. The trade-off between the goodness of fit (R^2) and the goodness of prediction (Q^2). One PLS component appears to be the optimal model, as this is where maximum Q^2 is seen, and difference between R^2 and Q^2 is reasonably small. After one PLS component, the Q^2 declines indicating that the model is being fitted to noise (random error).

Once the biomarker discovery experiment is completed, a short list of significant metabolites generated and a specific discriminant model built, then a receiver operating characteristic (ROC) curve can be created, based on the model predictions. ROC curves plot the sensitivity (true positive rate) versus the 1-specificity (false positive rate). Often, there is a trade-off between sensitivity and specificity, as one generally increases at the expense of the other. ROC curves are limited to binary outcome experimental designs such as case-control studies. In the context of metabolomics, where the outcome is continuous (concentrations of metabolites), then a specific concentration must be determined as the cut-point of a test. The cut-point forms the border between one outcome (case) versus another outcome (control). Essentially a ROC curve is a graph of the true positive rate versus the false positive rate for different decision boundaries or cut-points of a diagnostic test. The equal distribution line is a 45° line that connects the origin (0,0) to the point (1,1). If the area under the ROC curve (AuROC) is 0.5, the variable is distributed almost equally between cases and controls, such that any diagnostic test is essentially no better than chance. An AuROC of 1 means there is perfect classification of samples, as the test is 100% sensitive, 100% specific.[44, 62] An AuROC of ≥0.9 is considered an excellent test, and an AuROC of 0.8-0.9 is a good test.[65]

1.2.9 Metabolomics applications: from bench to bedside

The field of metabolomics has grown exponentially in the last fifteen years. The three basic categories of metabolomics studies in humans are:

diagnostic/prognostic purposes where usually predictive biomarkers of disease are sought; *pathogenesis* studies where biochemical pathways/mechanism of disease are investigated; and *risk factor* studies, where associations between the human metabolome and factors such as diet, lifestyle, environmental stressors are identified.[56] Metabolomics have a role in both oncological and non-oncological diseases. Numerous disruptions in carbohydrate, lipid and other biochemical pathways occur in cancer cell initiation and propagation, so studying metabolites that are either intermediates or end products of these dysregulated pathways may offer insight into new diagnostic and therapeutic targets for GC.

1.3 Summary

GC is a highly morbid and fatal disease. Screening methods are limited, and by the time of diagnosis, the disease is often in the advanced stages. As such, therapeutic options are limited. Metabolomics, the newest of the "omics" technologies, has shown promise in the area of surgical oncology. Although relatively new compared to more upstream methods such as genomics and transcriptomics, previous metabolomics studies of other malignancies have identified putative biomarkers that may be of use in disease diagnosis and prognosis. With further validation and experimentation, such biomarkers may form a disease specific profile that could be used as a screening test in the future. This program of research seeks to identify a disease specific metabolomic profile of GC from urinary samples using ^{1}H-NMR spectroscopy as the analytical platform. If such

a metabolomic signature is demonstrated, then this could have important implications for early screening of GC, and provide opportunities for earlier medical/surgical intervention, which in the long run, may reduce the morbidity and mortality associated with the disease.

1.4 Objectives

The objective of this program of research is to:

identify whether a disease specific urinary metabolomic profile is associated with GC compared to BN and HE using ^{1}H-NMR spectroscopy

1.5 Program of research

This thesis begins with a background discussion of GC and metabolomics. In Chapter 1 the first portion reviews the epidemiology, diagnosis, screening, therapy and prognosis of GC; this is followed by a review of metabolomics where the background and components of the metabolome workflow from study design to data analysis/interpretation are reviewed.

Chapter 2 is a comprehensive review of GC specific metabolomics (published in *World Journal of Gastroenterology*, Chan et al 2014). It summarizes the small number of recent studies that have been done in this area.

Chapter 3 compares the urinary metabolomic profile of a cohort of three types of patients: GC, BN gastric disease (portal hypertensive gastropathy,

gastroesophageal reflux disease, gastritis, gastric ulcers, non-cancerous gastric polyps, varices), and HE patients. There are distinct metabolites that separate GC from HE patients, but not the GC and BN patients due to phenotypic heterogeneity within the BN group. Gastritis patients appear to separate into two groups. Those that cluster with GC tend to have chronic gastritis, whereas those with mild superficial gastritis tend to cluster with HE. There is some misclassification, but these findings correlate with Correa's hypothesis on GC tumorigenesis from CAG to GC. As gastritis advances, it becomes metabolically and phenotypically more similar to GC. Validation with a larger sample of gastritis patients is necessary to observe if this parallel change is consistent.

Chapter 4 is the concluding chapter of this thesis and reviews the new information discovered from these experiments, as well as discusses future applications of metabolomics.

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CHAPTER 2: POTENTIAL ROLE OF METABOLOMICS IN DIAGNOSIS AND SURVEILLANCE OF GASTRIC CANCER

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Accepted for publication: 8 April 2014

Published online: 28 September 2014

2.1 Abstract

Gastric cancer is one of the deadliest cancers worldwide, and is especially prevalent in Asian countries. With such high morbidity and mortality, early diagnosis is essential to achieving curative intent treatment and long term survival. Metabolomics is a new field of study that analyzes metabolites from biofluids and tissue samples. While metabolomics is still in its infancy, there are numerous potential applications in oncology, specifically early diagnosis. Only a few studies in the literature have examined metabolomics' role in gastric cancer. Various fatty acid, carbohydrate, nucleic acid, and amino acid metabolites have been identified that distinguish gastric cancer from normal tissue and benign gastric disease. However, findings from these few studies are at times conflicting. Most studies demonstrate some relationship of cancer cells to the Warburg Effect, in that glycolysis predominates with conversion of pyruvate to lactate. This is one of the most consistent findings across the literature. There is less consistency in metabolomic signature with respect to nucleic acids, lipids and amino acids. In spite of this, metabolomics holds some promise for cancer surveillance but further studies are necessary to achieve consistency and validation before it can be widely employed as a clinical tool.

Key words: Gastric cancer; Metabolomics; Screening; Biomarkers; Surveillance

Core tip: There are differences in metabolomic profiles of gastric cancer patients and healthy controls, as well as between different stages of gastric cancer. The transition from normal to malignant consistently shows upregulation in lactate and downregulation of glucose consistent with the Warburg Effect. This trend is perpetuated as cells advance from non-invasive to invasive. Key tricarboxylic acid (TCA) cycle intermediates and amino acids are elevated as a result of anaplerotic reactions. Perpetuation of the TCA cycle generates energy for essential cell functions. There is less consistency between lipid and nucleic acid metabolites.

2.2 Introduction

The burden of gastric cancer is significant in Canada and worldwide. In 2013, the Canadian Cancer Society estimated there were 3300 new cases of gastric cancer which caused 3.3% of all male cancer related deaths, and 2.2% of all female cancer related deaths.[1] On a global scale, an estimated 990000 people were diagnosed in 2008, with 60% of those cases occurring in East Asia.[2] With an estimated 736000 deaths worldwide,[3] the fatality to case ratio is approximately 70%.[4] Despite these grim statistics, overall morbidity and mortality are declining due to changes in diet, treatment for *Helicobacter pylori*, early screening programs, improved surgical techniques and chemotherapy regimens.

Much of the mortality is attributable to delayed symptoms of gastric cancer. Early stage gastric cancer is asymptomatic: it takes an estimated 44 months to progress to an advanced stage.[5] Commonly patients present with vague epigastric pain, unintentional weight loss, anemia from occult blood loss, or dysphagia if the tumour is proximal. Gastric cancers that do not penetrate into the muscularis propria are asymptomatic in up to 80% of cases; occasionally, patients experience epigastric pain or “dyspepsia”. Dyspeptic symptoms occur in up to 40% of the population, so its value as a predictor of gastric cancer is limited. Furthermore, amongst those who have dyspepsia, previous studies have found that only 1%-2% of them will develop gastric cancer.[6-8] With such high morbidity and mortality, early

diagnosis is key. This review will highlight current surveillance methods and summarize how metabolomics may have important applications in future cancer surveillance and diagnosis.

2.3 Current surveillance methods

There are currently several methods of detecting gastric cancer, but no uniform screening guidelines. In Japan, where there is a high incidence of gastric cancer, screening has been introduced for everyone forty years of age and over. Since 1962, Japanese have employed barium-meal photofluorography as a screening test. The initial exam consists of a series of 8 X-rays. If this is abnormal, a detailed exam with 11 X-RAYs is undertaken. Endoscopy is then used to analyzed suspicious lesions identified on barium exam.[5] Case control studies suggest a 40%-60% decrease in gastric cancer mortality with photofluorography screening. The sensitivity of photofluorography is 60%-80% and specificity is 80%-90%. Studies indicate that survival rates of the screened group are 74%-80% compared to 46%-56% in the non-screened group.[9] Currently gastrofluorography is a Grade B recommendation.

Endoscopy is another tool used in gastric cancer surveillance. Its sensitivity ranges from 77%-84%.[9] It can identify superficial flat and non-ulcerative lesions that barium studies can miss.[5] In a Japanese study, detection of gastric cancer by endoscopy was 2.7 to 4.6 fold higher than with barium swallow. Endoscopy is versatile, as it allows clinicians to biopsy tissue, and perform endoscopic ultrasound to determine depth of invasion (tumour or T stage), should there be a lesion in the stomach. Despite these abilities, endoscopy has limitations in that it depends heavily on skills of the endoscopist and on availability of gastroscopy. Also it can be difficult to visualize early stage gastric cancers; the sensitivity is estimated to be 50%-60%. No studies have compared survival of gastric cancer patients between screened and non-screened groups. Therefore endoscopy has significant limitations as a screening technique, but currently it is still the best test available.

Since the 1990s, serum pepsinogen has been incorporated into gastric screening programs. Pepsinogen I and II are proenzymes of pepsin, which originate in gastric mucosa. These markers reflect morphological and functional status of the gastric mucosa and can act as a marker for chronic atrophic gastritis (CAG). CAG is regarded as a precursor of gastric cancer, especially the intestinal type.[10] In Japan, a serum pepsinogen (PG) test based on serum PG I level and PG I/II ratio have been used for screening. As mucosal atrophy increases, the level of PG I and thus the PG I/II ratio decreases.[11] Recent studies[10, 11] show that PG testing is useful at detecting early gastric cancers, especially in combination with barium X-ray. If either one or both of the two screening methods are positive, patients are referred for upper endoscopy. Cutoff values for serum PG tests are ≤ 50 ug/L and PGI/II ratio ≤ 3.0. These values detected gastric cancer in 0.28% of cases compared to 0.1% with barium X-ray. Early stage gastric cancer accounted for 100% of cancers detected by PG, 83% of cancers detected by barium X-ray, and 81% of cancers detected by both PG and X-ray. Eighty-nine percent of cancers detected by PG were intramucosal, compared to only 50% detected by barium X-ray. In this study[42], pepsinogen testing seemed to be useful in detecting small cancers arising from atrophic gastric mucosa.

2.4 Metabolomics in Cancer

Metabolomics is a relatively new area of study and the latest addition to the "omics" family of genomics, transcriptomics, and proteomics. The central dogma of molecular biology describes flow of biological information in a system from DNA to RNA to protein to metabolites. Different "omics" interventions play a part at different stages of this dogma to glimpse the inner workings of cell, tissue and organism. The metabolome of an organism consists of the entire collection of low molecular weight (<1500 Daltons) metabolites.[12] Metabolites are required for maintenance, growth and normal functioning of a cell. Mapping the metabolomic profile provides a global picture of the organism at a specific point in time under a specific set of conditions. For any given disease state, a small genomic change can be amplified many times at the metabolite level and quantitatively measured.

Metabolites in biological samples such as tissues, urine, saliva and blood plasma can be measured, and this allows researchers to identify specific metabolic pathways. Previous studies have demonstrated that metabolic activities of cancer cells are markedly different from that of healthy cells. Studying the metabolomic profile may help distinguish certain cancer biomarkers, and provide keys to early diagnosis.

Biofluids such as urine and blood are optimal samples to study, as they can be obtained through minimally invasive means. Profiles of these biofluids can be linked back to their genetic origins to provide a view of disease pathways. As metabolites are "downstream" entitities compared to genes, they reflect cellular conditions at the time of sampling and can be considered "endpoint markers" for disease. There are currently several technologies for analyzing the metabolome: nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), liquid and gas chromatography.

NMR utilizes a magnetic field. Spins of the atoms inside the tissue sample or fluid align themselves with respect to the magnetic fields. A radiofrequency pulse from the NMR machine elevates spins to a higher energy orientation. When the radiofrequency is turned off, spins undergo relaxation and release energy, returning to their original lower energy configurations. During this process, an NMR signal is emitted that can be detected by a computer system. A series of peaks are generated. Their positions are characteristic of certain known molecules. The NMR spectra of most metabolites have been identified and any new spectra can be identified in reference to available data. Liquid and gas chromatography are two separation techniques that rely on partitioning liquid or gas from a sample solution. Separation depends on the physical properties of the substance such as boiling point and solubility. As these chemicals are eluted off the column, they can be detected and quantified. Mass spectrometry is an analytical technique that identifies compounds based on their mass to charge ratio.

Each of the different analytical techniques has its benefits and drawbacks. A major advantage of NMR is its non-invasiveness and non-reliance on metabolite separation. Samples are not eluted off so they can be recovered for further analysis by chromatography or spectrometry. Sample preparation for NMR is simple, and

lends itself well to metabolite profiling of intact biofluids like culture medium or semi-solid samples like cells or tissue. However, a major disadvantage of NMR is its low sensitivity compared to chromatographic techniques.[13] A cross-platform comparison of metabolomic methods by Buscher *et al*[14] demonstrated that the three platforms of gas chromatography, liquid chromatography, and capillary electrophoresis were roughly equivalent in terms of sensitivity, and all superior to NMR.

Metabolomics has been studied with relation to numerous other cancers,[12, 15] including breast,[16] prostate,[17] lung,[17] colorectal,[18] pancreatic[19], esophageal,[20] ovarian,[21] bladder,[22] and renal cell carcinoma[23] but to date, very little has been studied in the area of gastric cancer. This review summarizes current available literature on gastric cancer metabolomics. As it is a relatively new field, there are only a few studies. Our findings are presented below.

2.5 Normal versus Malignant Metabolomic Signatures

A few studies in the literature have compared metabolomic profiles of gastric cancer patients with healthy controls. The type of biofluid or tissue they use varies between studies. This review organizes metabolites from each study into four main classes of biomolecules: carbohydrates, amino acids, lipids, and nucleic acids. Table 2-1 summarizes metabolites from each study by biolmolecular class.

2.5.1 Carbohydrate metabolism

Hu *et al*[24] implanted human gastric cancer cells into 24 immune deficiency mice. They were randomly divided into a metastasis group, non-metastasis group and a normal group. Urine of these mice was collected and gas chromatography/mass spectrometry was employed to identify a metabolomic profile. Two diagnostic models for gastric cancer and metastasis were constructed by principal component analysis (PCA). PCA is a way to visualize distribution of metabolites between different disease states. A point on a graph can be plotted for

each patient and the clustering of individual points represent similarities in metabolite profiles between samples. Ten metabolites were different between normal and cancer groups. Seven metabolites were different between metastasis and non-metastasis groups. On the PCA scores plot, the normal group and cancer group were scattered into different regions. Similarly the PCA plot showed differential scatter between non-metastasis and metastasis groups. Levels of TCA intermediates such as butanedioic acid, malic acid, and citric acid were elevated in gastric cancer mice, as were lactic acid levels. This could be attributed to the "Warburg effect" in that glucose is often converted into lactic acid in cancer cells.[25]

Hirayama *et al*[26] investigated metabolites in tumour tissue and compared this with adjacent normal tissue on twelve resected gastric cancer specimens. They quantified 95 metabolites involved in glycolysis, pentose phosphate pathway, TCA and urea cycles. Metabolites in normal stomach tissue and tumour tissue were not well separated on PCA plot, making two types of tissues less distinguishable. With regards to glycolysis and the TCA cycle, Hirayama found that glucose concentrations were much lower in tumour than in normal tissues. Also pyruvate was decreased, while lactate concentration was increased in tumour tissues indicating a higher reliance of cancer cells on anaerobic breakdown of pyruvate under hypoxic cell conditions. This lab group identified elevated levels of TCA intermediates specifically that of succinate, fumarate, and malate in malignant tissue. These findings correlated to ones from Hu *et al.*[24]

Song *et al* studied gastric cancer resections and compared the metabolomic profiles of the cancerous tissue matched to normal tissue at least 8 cm away on the specimen.[27] This group noticed an increase in metabolites of aerobic glycolytic pathways namely alpha ketoglutarate and fumaric acid. Across all studies, lactate was the most consistently elevated carbohydrate pathway biomarker (four of four[24, 26, 28, 29] studies) between the cancer and control groups. Likewise glucose was the most consistently depleted (two of two[26, 29] studies). Malate was the most consistently elevated TCA cycle biomarker (three of three[24, 26, 29] studies). Other carbohydrate pathway products showed inconsistencies.

2.5.2 Amino Acid Metabolism

Amino acids can be an alternative energy source, and can be generated through anaplerotic reactions, a process whereby intermediates in a metabolic pathway are replenished from biomolecules outside of the pathway. Glutamine is a prime example of an anaplerotic reaction. It is converted to glutamate and then into alpha-ketoglutarate, a TCA cycle intermediate.[30]

Wu *et al.* investigated gastric cancer mucosa in conjunction with adjacent normal mucosa.[31] Amino acids such as serine, phosphoserine, L-cysteine, L-tyrosine, glutamine, isoleucine and valine were elevated in gastric cancer specimens. These amino acids can be produced by diverting glycolytic intermediates down alternate biochemical pathways. Song *et al*[32] found that valine exhibited the greatest fold change in GC patients compared to controls. Overall, glutamine and valine were the most commonly recognized amino acids.

2.5.3 Fatty acid metabolism

Cancerous cells are known to have dysregulation of fatty acid beta-oxidation and cell membrane synthesis. Hu *et al*[24] who studied human gastric cancer in mice models identified elevated levels of hexadecanoic acid and glycerol in cancerous compared to normal tissues. They interpreted this as upregulation of adipocyte lipolysis and elevated circulation levels of adipocyte hormone sensitive lipase. Song *et* al[27] found that squalene (an intermediate in cholesterol synthesis) was the most extensively depleted metabolite in gastric cancer specimens. Overall, there is great heterogeneity of lipids across studies.

2.5.4 Nucleic acid metabolism

The literature on nucleic acid metabolites is conflicting. Several studies reports that uric acid, the final metabolic product of purines is upregulated.[24, 33] Other purines

such as hypoxanthine[31] and guanosine[26] are generally elevated. This is in contrast to Aa's study[29] which showed decreases in uridine, an RNA building block.

Table 2-1: Marker metabolites between gastric cancer and healthy controls

Ref.	Animal *vs* human analytical platform	Sample type groups	Statistically significant metabolites identified ($P < 0.05$) (up or down indicate levels in cancer group)
Hu *et al*[24], 2011	Animal (mice) Gas chromatography, Mass Spectrometry	Urine Metastasis group ($n = 8$) Non-metastasis group ($n = 8$) Normal group ($n = 8$)	Carbohydrates: Anaerobic respiration: lactate up TCA cycle: citric acid up, malic acid up Nucleic acids: uric acid up Lipids: hexadecanoic acid up Others: butanoic acid, propanoic acid, glycerol, pyrimidine, glycerol all up
Hirayama *et al*[26], 2009	Human Capillary electrophoresis Mass spectrometry	Resected stomach specimens ($n = 12$) GC tissue Adjacent normal tissue	Carbohydrates: Anaerobic respiration: lactate up Glycolysis: glucose down TCA cycle: citric acid down, malic acid/fumarate up Amino acids: 19 elevated, except glutamine (no change) Nucleic acids: GMP up
Song *et al*[27], 2011	Human Gas chromatography, Mass spectrometry	Resected stomach specimens ($n = 30$) GC tissue Adjacent normal tissue	Carbohydrates: TCA cycle: fumarate up, alpha-ketoglutarate up Lipids: up: xylonic acid, octadecanoic acid Down: 9-hexadecanoic acid, *cis*-vaccenic acid, arachidonic acid, 9-octadecenamide, squalene Others: up: valeric acid, benzenepropanoic acid, 1-phenanthrene-carboxylic acid down: 3-hydroxybutanoic acid
Cai *et al*[28], 2010	Human Gas chromatography, Mass spectrometry	Resected stomach specimens ($n = 65$) GC tissue Adjacent normal tissue	Carbohydrates: Anaerobic respiration: lactate up Glycolysis: up: fructose, glyceraldehyde, pyruvate TCA cycle: up isocitric acid, fumarate down
Aa *et al*[29], 2012	Human Gas chromatography, mass spectrometry	Plasma and tissue Pre-op GC ($n = 17$) Post-op GC ($n = 15$) CSG ($n = 20$)	Tissue samples show larger variations between GC and CSG than plasma samples (up/down show change for tissue CG compared to tissue CSG) Carbohydrates: Anaerobic respiration: lactate up Glycolysis: glucose down, fructose-6-phosphate down TCA cycle: citrate up, malate up, fumarate up Other: maltose down, ribose down, glyceric acid-2,3-diphosphate down Amino acids: cysteine up Lipids: up: docosahexanoic acid, heptanoic acid, 9-Z-hexadecenoic acid, beta-hydroxybutyrate down: cholesterol Nucleic acids: uracil up, uridine down Others: up: 2-aminoadipate, monomethylphosphate down: inositol, ribitol, beta-*D*-methylglucopyranoside
Wu *et al*[31], 2010	Human Gas chromatography, Mass spectrometry	Resected stomach specimens ($n = 18$) GC tissue Adjacent normal tissue	Carbohydrates: up: galactofuranoside down: *L*-altrose, *L*-mannofuranose, *D*-ribofuranose Amino acids: up: *L*-valine, *L*-isoleucine, serine, *L*-glutamine Down: phosphoserine Others: up: heptanedioic acid, propanoic acid, phenanthrenol, butanetriol, acetamide, butenoic acid, oxazolethione, naphthalene down: myo-inositol
Song *et al*[32], 2012	Human Gas chromatography, Mass spectrometry	Serum GC ($n = 30$) Healthy ($n = 30$)	Carbohydrates: down: fumarate, 2-*O*-mesyl arabinose Amino acids: up: valine, sarcosine Down: glutamine, hexanedioic acid Lipids: down: 9, 12-octadecadienoic acid, 9-octadecenoic acid, trans-13-octadecenoic acid, nonhexacontanoic acid Cholesterol: up cholesta-3,5-diene, cholesterol, pentafluoropropionate, cholesterol, cholest-5- en-3-ol
Ikeda *et al*[36], 2012	Human Gas chromatography, Mass spectrometry	Serum GC ($n = 11$) Healthy ($n = 12$)	Carbohydrates: Glycolysis: down: pyruvate Others: up: 3-hydroxypropionic acid Down: 3-hydroxyisobutyric acid, octanoic acid, phosphoric acid
Kim *et al*[38], 2010	Animal (mice) ^{1}H-NMR	Urine GC ($n = 10$) Healthy ($n = 10$)	Carbohydrates: TCA cycle: Citrate up, 2-oxoglutarate up Others: up: 3-indoxylsulfate Down: taurine, trimethylamine, oxaloacetate, TMAO, hippurate
Miyagi *et al*[41], 2011	Human Liquid chromato-graphy, mass spectrometry electrospray ionization	Serum GC ($n = 199$) Healthy ($n = 985$)	Amino acids only: up: serine, glutamine, ornithine, proline down: asparagine, valine, methionine, tyrosine, histidine, tryptophan, phenylalanine, leucine

CSG: Chronic superficial gastritis; GC: Gastric cancer; GMP; Guanosine monophosphate; ^{1}H-NMR: Proton nuclear magnetic resonance spectroscopy; TCA: Tricarboxylic acid cycle; TMAO: Trimethylamine-*N*-oxide.

2.6 Metabolomic profile and stage

While it is interesting to see differences in metabolomic profile between normal and cancerous tissue, it is also useful to examine how the profile evolves along a gradient as it goes through the benign to dysplastic to cancerous sequence. In the 1980s, Correa proposed a model of human intestinal-type gastric carcinogenesis from normal mucosa to chronic superficial gastritis (CSG), to CAG, to intestinal metaplasia (IM) to dysplasia (DYS) and then to intestinal-type GC.[34] Yu and colleagues[33] employed gas chromatography and time-of-flight mass spectrometry to determine metabolite levels in plasma of 80 patients with the spectrum of disease described previously by Correa. They found that the metabolic phenotype of CSG is significantly different from GC, while that of IM is similar to GC. Knowing metabolites of each stage of the progression to GC, may be used as markers to indicate a risk for malignancy. Yu *et* al[33] also found that when they mapped metabolites identified in GC, it was not much different from postoperative GC specimens within a 4-6 week window. Perhaps this is because it takes longer for metabolic derangements to resolve. Key metabolic differences between different histological stages are summarized on Table 2-2.

Yu et al also found significant differences in serum levels of amino acids between GC and CSG patients. Levels of three amino acids- glutamate, cysteine, and glycine were upregulated. These amino acids are building blocks for glutathione synthesis, which is an important anti-oxidant. 2-hydroxybutyrate, which is postulated to be a by-product in glutathione synthesis was also elevated, as were asparagine and ornithine. Most other amino acids did not show an increase in this study, unlike previous studies on gastric cancer tissue.[26] This shows that metabolomic profiling in blood may be different than in tissue. Lipid synthesis was similar between CSG and GC, except 11-eicosanoic acid and azelaic acid, which were elevated in malignant samples. Postoperative GC patients had decreased levels of urate, the end product of purine catabolism. This suggests that growth and DNA proliferation is slowed once tumour is resected.

It is also interesting to note how metabolomic profile changes with increasing TNM stages. Song and colleagues[27] did not notice any significant variation in metabolites as patients progressed through T stage. They postulated that either metabolic perturbations may not be directly associated with pathological stages, or that the platform of gas chromatography and mass spectrometry is not sufficiently sensitive to identify metabolite changes. On the other hand, Wu *et al*[31] identified that as cancers became more invasive (T3/T4 stage), there was a simultaneous increase in amino acids L-cysteine, hypoxanthine, L-tyrosine, as well as a decrease in levels of phenanthrenol and butanoic acid. Chen *et al*[35] found that proline was the most upregulated amino acid from non-metastatic to metastatic specimens (2.45 fold increase), while glutamine was the most downregulated amino acid (1.71 fold).

Apart from amino acids, other biomolecules show changes between stages. Ikeda *et al*[36] studied the sera of eleven GC patients and found that 3-hydropropionic acid and pyruvic acid, the terminal product of glycolysis, marked the greatest separation between healthy and cancer patients. In Stage I GC, there was a 1.5 fold increase in levels of 3-hydropropionic acid and 0.7 fold decrease in pyruvic acid compared to healthy controls. Both values were only statistically significant in Stage I cancers. This may have some future utility in diagnosing GC early, but more studies validating similar findings will be necessary. Key metabolic differences between different stages are highlighted in Table 2-2.

2.7 Metabolomic profile and proximal gastric cancer

Over the last twenty to thirty years, there has been an increase in the numbers of proximal stomach tumours. As of 2011, gastroesophageal (GE) tumours affect 1.5 million people per year worldwide and contribute to 15% of cancer related deaths. The 5-year survival rate for localized tumours is 34%, while for all stages combined it is only 17%.[37] Given the poor prognosis of these proximal tumours, some recent metabolomic studies look at the unique profile of cardia and GE tumours in the hope of shedding light on early diagnostic possibilities.

Cai *et al*[28] used a combined proteomics and metabolomics approach to investigate gastric cardia cancer. They found that there was a dysregulation of pyruvic acid efflux in development of cardia cancer. A transition from glycolysis to the Kreb's cycle was associated with cancer inhibition. Several biomarkers related to glucose metabolism were elevated in cardia cancer samples compared to non-cancerous cardia tissue. Five enzymes from glycolysis were upregulated while five enzymes involved in Kreb's cycle and oxidative phosphorylation were downregulated in malignant samples. Several intermediates in glucose metabolism were identified in higher concentrations in gastric cancer samples including fructose, glyceraldehyde, pyruvic acid and lactate. A higher level of pyruvic acid was transformed into lactic acid, rather than acetyl CoA following Krebs cycle. These results suggest that glycolysis followed by anaerobic respiration were the major biochemical pathways to metabolize glucose in cardia cells, whereas Krebs cycle and oxidative phosphorylation were impaired. This is consistent with previous studies validating the Warburg effect.

A 2013 systematic review by Abbassi-Ghadi summarized metabolomic findings on gastroesophageal cancer.[37] Twenty studies (11 tissue, 8 serum, 1 urine and 1 gastric content) were included. They classified metabolites into cellular respiration, proteins, lipids and nucleic acids. The most commonly recognized metabolites of the tricyclic acid cycle were lactate and fumarate. Valine, glutamine, and glutamate are the most commonly identified amino acid biomarkers. Most metabolites have shown contradictory results in terms of abundance between cancer and control groups, although there is a general trend of upregulation of amino acids. Amongst all tissues, glutamine is the most consistent biomarker of GE cancer as it is upregulated in serum, urine and tumour tissues.

Sulphur containing compounds, from either incomplete metabolism of methionine in the transamination pathway or by bacterial metabolism, were also upregulated in cancer patients. In terms of lipid metabolites, myo-inositol, and cell membrane constituents choline, and phosphocholine were elevated. Of the endogenous ketones acetone and beta-hydroxybutyrate, have been described as potential biomarkers of GE cancer. Nucleotide metabolites in esophageal cancer

studies report increased levels of pyrimidines via gas chromatography-mass spectrometry and increased adenine and uridine with high resolution-magic angle spinning-NMR (HR-MAS-NMR).

Table 2-2: Marker metabolites between stages of gastric cancer

Ref.	Animal *vs* human platform utilized	Sample type groups	Statistically significant metabolites identified ($P < 0.05$)
Hu *et al*[24], 2011	Animal (mice) Gas chromatography, mass spectrometry	Urine Metastasis group (n = 8) Non-metastasis group (n = 8) Normal group (n = 8)	Up/down for metastasis group compared to non-metastasis: Amino acids: alanine, *L*-proline, *L*-threonine all down Others: butanoic acid down, glycerol down, butanedioic acid up, myo-inositol up
Song *et al*[27], 2011	Humans Gas chromatography, mass spectrometry	Resected stomach specimens (n = 30) Gastric Cancer tissue, Adjacent normal tissue	Did not find metabolite differences between TNM stages
Wu *et al*[31], 2010	Human Gas chromatography, mass spectrometry	Resected stomach specimens (n = 18) Gastric Cancer tissue -T1/T2 stage (n = 5) -T3/T4 stage (n = 13)	Up/down for T3/T4 metabolites compared to T1/T2 Amino acids: *L*-cysteine, *L*-tyrosine both up Nucleic acids: hypoxanthine up Others: butanoic acid, phenanthrenol both down
Yu *et al*[33], 2011	Human Gas chromatography, mass spectrometry	Plasma (n = 80) CSG (n = 19) CAG (n = 13) IM (n = 10) DYS (n = 22) Pre-op GC(n = 9 pre-op) - 4-6 wk post-op (n = 13)	Up/down for pre-op GC compared to CSG: Carbohydrates: threonate down Amino acids: up: ornithine, pyroglutamate, glutamate, asparagine Lipids: 11-eicosenoic acid up Nucleic acids: urate up Other: up: 1-monohexadecanoyl-glycerol, gamma-tocopherol, 2-hydroxybutyrate, azelaic acid
Chen *et al*[35], 2010	Animal (mice) Gas chromatography, mass spectrometry	GC metastasis group (n = 8) GC non-metastasis group (n = 8)	Up/down for metastasis group compared to non-metastasis: Carbohydrates: Anaerobic respiration: lactate up Glycolysis: glucose down TCA cycle: malic acid up, succinate down Amino acids: up: alanine, glycine, valine, proline, serine, leucine, dimethylglycine, aspartic acid, phosphoserine, glutamate, lysine, arginine down: isoleucine, methionine, threonine, glutamine Nucleic acids: hypoxanthine down, pyrimidine up Others: up: propanedioic acid, pyrrolidine, inositol, docosanoic acid, octadecanoic acid down: propanamide, butanedioic acid
Ikeda *et al*[36], 2012	Human Gas chromatography, mass spectrometry	Serum GC (n = 11) Stages I -IV	3-hydroxypropionic acid up, pyruvic acid down (statistically significant only in Stage I GC)

CAG: Chronic atrophic gastritis; CSG: Chronic superficial gastritis; DYS: Dysplasia; GC: Gastric cancer; IM: Intestinal metaplasia; T1/T2/T3/T4: Tumour stage 1/2/3/4; TCA: Tricarboxylic acid cycle; TNM: Tumour node metastasis classification.

2.8 Discussion

This review demonstrates that there are significant inconsistencies in the relative abundance of metabolites between not only gastric cancer and controls, but also amongst various stages of cancer. Metabolites upregulated in one study may be downregulated in another. This may be attributable to analytical technique

(GC/MS/NMR), sample choice (blood/urine/tissue), or type of subject (animal/human).

Of the four types of biomolecules, carbohydrates are most consistent in terms of type and quantity of metabolites. Glucose was consistently downregulated. This may be due to upregulation of glycolysis, high consumption by cancer cells and diminished delivery from structurally and functionally defective blood vessels. Lactate was consistently elevated across all studies. This observation is in keeping with the Warburg effect.

In 1924, Otto Warburg observed that most cancer cells produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol. This occurs even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation via the TCA cycle. Scientists have called this phenomenon "anaerobic glycolysis". Healthy cells, in contrast, exhibit a lower rate of glycolysis followed by aerobic oxidation of pyruvate in mitochondria.[25] Metabolic differences observed by Warburg adapts cancer cells to the relatively hypoxic environment inside solid tumours. He originally postulated that there was a mitochondrial defect impairing aerobic oxidation; however, subsequent studies have shown that most cancer cells have normal mitochondria. Anaerobic glycolysis is an inefficient way to produce energy in the form of adenosine triphosphate (ATP), and the reason as to why cancer cells predominantly utilize this method is still under study. Most studies have identified metabolites in glucose utilization and some kind of connection to the Warburg effect.

Despite this, there are still elevated levels of certain TCA cycle intermediates, including malate (elevated in three of three studies[24, 26, 29]), citrate (elevated in three[24, 29, 38] of four[26] studies), and fumarate (elevated in three[26, 27, 29] of five[28, 32] studies). While this may seem contradictory to the Warburg effect and cancer cell's preference for anaerobic reactions, these TCA cycle intermediates may be funneled in from anaplerotic reactions rather than elevated TCA cycle activity. Glutamine is one example of such. It is an essential nitrogen donor for several key metabolic enzymes and for the de novo synthesis of nucleic acids[39]. Glutamine is converted to

alpha-ketoglutarate, which is a TCA intermediate; continuation of this cycle generates additional energy to produce building blocks for cells.

Amino acid metabolism demonstrated variations as well, but glutamine and valine were most commonly elevated across studies. Like glutamine, valine is essential as an anaplerotic substrate. Valine is a branched chain amino acid that can be oxidized into succinyl Co-A, another TCA cycle intermediate.[40] Other TCA intermediates include fumarate, citrate, and alpha-ketoglutarate, which are points in the cycle where amino acids can feed in.

Lipid metabolites have been inconsistent, although squalene, an intermediate in cholesterol synthesis, was downregulated. Cholesterol is an essential component of cell membranes. Squalene depletion may be a sign of excess demand for cell membrane synthesis. Although cancer cells are known to replicate quickly, it is interesting that nucleic acid metabolites do not show a consistent upregulation. Hirayama[26] inferred that cancer cells have a growth advantage over their normal counterparts, by utilizing alternative pathways such as anaerobic glycolysis, glutaminolysis, autophagic production of amino acids instead of securing more ATP and other building blocks for DNA synthesis.

For any given study, numerous metabolites were different between stages, but across studies, there were few consistencies. Similar to changes that occurred between normal to cancerous groups, a transition from non-metastatic to metastatic showed persistent elevations in lactate, malate and glutamate with a decrease in glucose.[33, 35] This may indicate that the Warburg Effect and anaplerotic reactions are still major contributors to the sustenance of metastatic cell lines.

The articles in this review have several limitations that may account for inconsistencies in metabolites. As previously mentioned, there are differences in analytical platform and different sensitivities for detection of such metabolites across different studies. Metabolomics is a relatively new field, and as such, the techniques are not yet standardized. Also several studies had a small sample size ($n \leq 30$ per group). This increases the possibility of chance findings and diminishes power of the study. The examination of mice versus human metabolites could be another source of error. Although human gastric cell lines were implanted into mice,

human physiology is still considerably more complex; this may account for differences between human and animals studies.

Some studies matched for age and gender between groups (Song[32]) but others (Ikeda[36]) just used twelve human volunteers. This introduces selection bias. The small sample size and lack of age and gender matching between cancer and normal groups could confound the metabolomic profile. Depending on the type of tissue or biofluid sampled, there may also be differences. Aa[29] noted dissmilarities in relative quantities between tissue and serum in their study between GC and CSG patients. For example, TCA intermediates, lactate, amino acids and free fatty acids were more abundant in tissues than in the patient matched sera. This suggests that metabolism is most intensive at the tissue level and becomes somewhat diluted in biofluids.

2.9 Conclusion

Gastric cancer is the one of the leading causes of cancer deaths worldwide, and is especially prevalent in Asian countries like Japan, China and Korea. Current surveillance techniques such as barium photofluorography, endoscopy and serum pepsinogen testing are known to have limitations. As of late, metabolomics is a new area of study that has joined the armamentarium of diagnostic possibilities. Only a handful of studies have looked at the role of metabolomics in gastric cancer. Variations in fatty acid, carbohydrate, lipid and nucleic acid metabolites have been identified that distinguish cancerous from healthy individuals, as well as stage of gastric cancer. Aberrations in carbohydrate metabolism seem to be the most preserved feature of these metabolic studies, as well as elevation of key amino acids that contribute to carbohydrate pathways through anaplerotic reactions.

In spite of the differences identified, there are inconsistencies in metabolomic profiles between studies. This may be attributable to differences in sample type, as plasma compared to urine compared to stomach tissue may yield different metabolomic profiles, as well as sampling techniques, analytical platforms

and subject type (animal or human). While these early studies on metabolomics show promise, this is a relatively new field in the pre-clinical phase. Our lab group is currently studying metabolic differences in urine between Stage I-III gastric cancer patients, benign gastric disease and healthy controls, as well as how *Helicobacter pylori* affects the metabolic signature. NMR spectroscopy will be employed. This future research will hopefully add to the growing body of knowledge and advance the clinical applicability of metabolomics in surveillance and diagnosis of gastric cancer.

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CHAPTER 3: ^{1}H-NMR URINARY METABOLOMIC PROFILING FOR DIAGNOSIS OF GASTRIC CANCER

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3.1 Abstract

Background

Gastric adenocarcinoma causes significant morbidity and mortality. Current screening modalities have limitations. Metabolomics has shown some promise in early cancer diagnosis. This study sought to identify whether gastric cancer (GC) has a unique urinary metabolomic profile compared to benign gastric disease (BN) and healthy (HE) patients.

Methods

Midstream urine samples from 43 GC, 40 BN, and 40 matched HE patients were biobanked at -80°C. Samples were chemically analyzed using ^{1}H nuclear magnetic resonance spectroscopy, generating 77 reproducible metabolites. Univariate and multivariate (MVA) statistics were employed. A parsimonious biomarker profile of GC was investigated using LASSO regularized logistic regression (LASSO-LR). Receiver operating characteristic (ROC) curves were used to assess model performance.

Results

Twenty-eight metabolites differed significantly between GC and HE, of which 10 also differed between BN and HE. Using MVA, GC displayed a clear discriminatory biomarker profile; the BN profile overlapped with GC and HE. LASSO-LR identified three discriminatory metabolites: 2-hydroxyisobutyrate, 3-indoxylsulfate, and alanine, which produced a discriminatory model with an area under ROC of 0.95.

Conclusions

GC patients have a distinct urinary metabolite profile compared to HE controls and a subset of BN patients. This preliminary study shows clinical potential for metabolic profiling for early GC detection.

Keywords

Biomarkers, metabolomics, screening, gastric cancer, nuclear magnetic resonance

3.2 Introduction

Gastric adenocarcinoma (GC) is the fifth most common cancer worldwide and the third most deadly. Approximately one million people are diagnosed worldwide every year, and there is a 70% mortality rate.[1,2] Premature death and disability from GC have a large impact on society. In lower middle income countries (ranked by gross domestic product- GDP), there were close to 4.8 million disability adjusted life years (DALY) lost in one year due to GC.[3] Sixty percent of cases occur in East Asia, with Korea, Japan and China having the top three prevalence rates.[4] GC is often

diagnosed late, as non-specific symptoms such as dyspepsia resemble benign (BN) causes such as gastritis. In spite of this, cancers identified early have a moderate chance of cure. The five-year survival rate of Stage IA tumours is 71% and Stage IB tumours is 57%.[5] This highlights the importance of appropriate screening in higher risk populations. Current screening tools include endoscopy, barium swallow, and serum pepsinogen testing, but each have their limitations.

Metabolomics is the study of low molecular weight chemicals (<1500 Da) in a biological system and is increasingly utilised in the area of oncology.[6-8] It is the most downstream of the "omics" sciences (Genomics, Transcriptomics, Proteomics, etc.), and is considered closest to an organism's phenotype.[9] Altered metabolism is one of the features of cancer propagation. Malignant cells can evade regular apoptotic checks and balances[10] and solid tumours in particular demonstrate enhanced ability to ferment glucose into lactate, allowing them to thrive in hypoxic environments.[11] Changes in biochemical pathways provide cancer cells with new adaptations to propagate, so studying the metabolites of such perturbed pathways may offer insight into new diagnostic and therapeutic targets for GC. Identification of a distinct metabolomic profile of GC could be the basis of a non-invasive screening tool in targeted, high-risk populations.

There are relatively few studies in the area of GC metabolomics. Several of the studies are serum[12-14] or tissue based.[15] Most studies have a case control design comparing GC vs HE;[14,16,17] however, in real life clinical situations, healthy people will not be seeking medical attention as they do not have symptoms. This experiment added the benign (BN) disease group to test whether GC urinary

metabolomic profile differed not only from HE, but also with respect to BN gastric disease using ^{1}hydrogen nuclear magnetic resonance (^{1}H-NMR) spectroscopy as the analytical platform. Due to overlapping symptoms of benign and malignant disease, a biomarker model discriminating GC and BN conditions may be of greater clinical utility than distinguishing GC and HE. Urine was selected as the biofluid because it is economical, non-invasive and requires minimal sample processing before chemical analysis. All three groups were age, sex, and body mass index (BMI) matched. It is hypothesised that GC patients have a distinct set of metabolites (a biomarker profile) that can be discriminated from BN and HE patients.

3.3 Materials and Methods

Patient selection

Samples were collected between January 2009 and December 2014 from three hospitals in Edmonton, Alberta, Canada (the Royal Alexandra Hospital, University of Alberta Hospital, and Cross Cancer Institute). Ethics approval was obtained from the Health Research Ethics Board at the University of Alberta. A convenience sample of 43 GC, 40 BN, and 40 HE was obtained. In patients with malignancy, samples were collected prior to chemoradiotherapy and surgery. All patients provided written informed consent to collect and utilise midstream urine samples for research purposes.

Inclusion criteria for cancer patients were: biopsy confirmed diagnosis of GC, age ≥ 18 years old, and no metastases on their staging computed tomography (CT)

scans. BN patients had to experience gastrointestinal symptoms (such as haematemesis or epigastric discomfort), and must have endoscopic evidence within the last six months of consent that symptoms were not due to a malignant cause. BN and HE patients were recruited from August to November 2013 from the same hospitals as GC cases. Groups were matched on age, gender and BMI. BN patients had any of the following conditions: gastritis, gastro-oesophageal reflux disease (GORD), portal hypertensive gastropathy, varices, gastritis, ulcers, and polyps. HE controls did not have any declared history of cancer, and did not experience any gastrointestinal symptoms.

Exclusion criteria included: breastfeeding, pregnancy, significant cardiac disease with New York Heart Association ≥ Class II (documented myocardial infarction within 6 months, unstable angina, or history of congestive heart failure), uncontrolled bacterial, viral or fungal infection, and prior history of cancer. Those with clinically significant renal disease (glomerular filtration rate < 30 mL/min) were excluded, as impaired urinary metabolite excretion could be a confounder when determining metabolomic profile. Patients were enrolled if they satisfied all inclusion/exclusion criteria.

Sample collection and storage

Midstream urine samples were collected and processed within two hours of sample provision. The resulting aliquots were biobanked at -80°C until preparation for NMR analysis. Disease class and patient identification was anonymized before chemical analysis to remove the possibility of analysis bias. Samples were thawed

to make 1 mL aliquots of urine mixed with 50 µL of 0.42% sodium azide preservative.

Sample preparation for NMR spectroscopy

The experimental run order was block randomised, such that each block randomly contained 1 GC, 1 BN and 1 HE sample. The experiment was divided into four batches and performed over a ten-day time span. To assess precision and repeatability of metabolite quantification, a quality control sample (QC) consisting of multiple aliquots derived from a single HE patient's urine was analysed every tenth sample.[18] Seventeen QCs were used in the experiment.

Urine aliquots were thawed and prepared by adding 75 µL of a chemical shift standard (Chenomx Inc., Edmonton, Alberta, Canada) containing 4.6 mM 2,2-dimethyl-2-silapentane-5-sulfonate-d6 sodium salt (DSS-D6), 0.20% w/v NaN_3 and 98.0% v/v D_2O, to 675 µL of urine. Samples were titrated to a final pH of 6.75 ± 0.05 using small volumes of sodium hydroxide (NaOH) and hydrochloric acid (HCl). Samples were centrifuged for 10 minutes at 10000 x g at 4 °C to remove particulate matter. Next, 700 µL of supernatant was transferred to a 5 mm diameter NMR tube (Wilmad, Nuena, NJ, USA) immediately prior to NMR acquisition.

^{1}H-NMR Spectroscopy and NMR Data Processing

All one-dimensional (1D) ^{1}H-NMR spectra were acquired at Canada's National High Field Nuclear Magnetic Resonance Centre using a 600 MHz Varian Inova spectrometer equipped with a 5 mm inverse proton (Hx) probe with z-axis

gradient coil. The entire dataset was collected at 25°C using the first increment of a 2-dimensional-^{1}H,^{1}H-NOESY, with a recycling delay (d1) of 10 ms followed by a water presaturation delay of 900 ms at 6 dB, a 100 ms NOE mixing time, a 4 s acquisition time and a spectral width of 7200 Hz. A total number of 128 transients were collected for each sample. The free induction decays were processed zero-filled to 128K points before Fourier transform, and the produced spectra were line broadened by 0.2 Hz, phased, and baseline corrected. Reference deconvolution using the DSS methyl peak as was then applied to remove Voigt-profile line shapes and produced pure Lorentzian peaks.

Semi-targeted metabolite identification and quantification of 1D spectra was achieved using the 600 MHz database provided in Chenomx NMR Suite Professional software v7.6 (Chenomx Inc., Edmonton, Alberta, Canada).

Data Modeling and Statistical Analysis

First, probabilistic quotient normalized[19] was performed to correct for differences in sample metabolite dilution. Then, for each metabolite, the QC's relative standard deviation (QC-RSD) was calculated. In this biomarker discovery experiment, a QC-RSD <20% was sufficiently precise.[9] Any metabolites with QC-RSD above this threshold, and any metabolites that were detected in <15% of samples were not suitably reproducible for further consideration as effective biomarkers, and excluded from subsequent statistical analysis. This resulted in an abbreviated data matrix of 77 metabolite concentrations for each of 123 patients.

For the pairwise comparisons GC vs. HE and BN vs. HE, the null hypothesis of no difference in median metabolite concentrations was tested using the non-parametric Mann-Whitney *U* test. Correction for multiple comparisons was performed using the method described by Benjamini and Hochberg.[20] Both p-values and q-values are reported, as are median concentrations and median-fold differences for each pairwise comparison. To compare univariate statistical results from two arms of this study (GC vs HE, and BN vs HE) a biplot of log median fold change for metabolites significant in either comparison was constructed.

To investigate the potential utility of combining multiple metabolites into a single predictive model, multivariate statistical analysis was performed using SIMCA software (version 13, Umetrics, Umea, Sweden). Original metabolite concentrations were log transformed in order to stabilize variance and then each metabolite vector was mean-centered and scaled to unit variance to equalize metabolite concentration bias.[1] Principal Component Analysis (PCA)[22] was performed to identify outliers and check multivariate QC consistency.[18] Partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis, OPLS-DA[21] were then performed to generate appropriate multi-class, and binary class, discriminant models respectively. Seven-fold cross validation was used to optimize each model. For each model *Variable Importance in Projection* (VIP) scores were obtained for individual metabolites. VIP scores indicate the relative importance of each metabolite in a given PLS model. Metabolites with a VIP >1 are most influential in a model and thus contribute most to discriminating disease groups.[21]

Logistic regression optimized by LASSO regularization, (LASSO-LR) was then performed to derive an effective, robust, yet parsimonious discriminant GC biomarker model. Logistic regression is a type of probabilistic statistical classification model commonly used for predicting the outcome of a categorical dependent variable (in this case GC vs. HE), and can be considered as a special case of a generalized linear model (GLM) with the form: $logit(p_i) = \beta_0 + \beta_1 x_{1,i} + \beta_2 x_{2,i} \ldots + \beta_m x_{m,i}$ (where, p_i is the predicted probability of positive classification for the i^{th} patient; $x_{1,i} \ldots x_{m,i}$ are the m metabolite measurements for the i^{th} patient; β_0 is the regression constant; $\beta_1 \ldots \beta_m$ are regression coefficients indicating the relative influence of a particular metabolite on the outcome).

LASSO regularization[23] is a GLM variable selection method based on penalizing variables (metabolites) with low β values by forcing them to zero dependent on a regularization parameter λ. Optimization of λ was performed using 5-fold cross-validation with 100 Monte Carlo repetitions to ensure the avoidance of "over fitting" (i.e. ensuring the model is generalizable for future testing with new independent samples). The trade-off between number of variables and classification success is then determined *ad-hoc*. The resulting optimal classifier models were assessed using receiver operator characteristic (ROC) curve analyses. This allows determination *a posteriori* of the optimal "decision boundary" (the predictive score determining whether a sample is classified as case rather than control) and the associated optimal classification sensitivity and specificity. The area under the ROC curve (AuROC) is used as a generalized non-parametric estimate of biomarker utility (AuROC = 1 implies a perfect classifier; AuROC = 0.5 implies a

model which is no better than flipping a coin to determine outcome). Bootstrap resampling was performed (n=500) to estimate the 95% confidence interval (CI) for both the AuROC, and a model's optimal sensitivity given a fixed specificity. Additionally, all available data (GC, BN, HE) were projected through this model to quantify disease discrimination. Statistical analyses were performed with MatLab scripting language (MathWorks Inc., Natick, Massachusetts, United States), SIMCA (version 13, Umea, Sweden), and STATA Version 13 (College Station, TX: StataCorp LP).

3.4 Results

Patient Characteristics

Patient and tumour characteristics at baseline are listed in Table 3-1.

Table 3-1: Baseline characteristics of study subjects and tumour

Table 1. Baseline characteristics of study subjects and tumour			
Characteristic	**BN**	**GC**	**HE**
Number of patients	40	43	40
Mean Age (SD)	63.1 (9.0)	65.2 (12.0)	63.2 (8.8)
Gender (Male/Female)	19/21	28/15	23/17
Mean BMI (SD), in kg/m^2	29.5 (6.4)	27.6 (6.9)	27.7 (4.7)
***Helicobacter pylori* status (on biopsy or urea breath test)**			
Positive/Negative/Unknown	3/21/16	7/26/10	-
Benign Condition			
Gastritis only	13 (32.5%)	-	-
Ulcer only	4 (10.0%)	-	-
Gastritis & ulcer	1 (2.5%)	-	-
Gastritis & PHG	1 (2.5%)	-	-
Portal hypertensive gastropathy (PHG)	9 (22.5%)	-	-
Gastroesophageal reflux disease (GERD)	3 (7.5%)	-	-
varices	1 (2.5%)	-	-
polyps	5 (12.5%)	-	-
reactive gastropathy	1 (2.5%)	-	-
normal scope with GI symptoms	2 (5.0%)	-	-
Overall TNM stage			
Ia/b	-	3/3	-
IIa/b	-	8/3	-
IIIa/b/c	-	2/5/3	-
IV	-	14	-
Unknown	-	2	-
Tumour location			
GE junction/Cardia/Fundus/Body/Antrum/Pylorus	-	6/1/4/15/16/1	-
Lauren histological class			
Diffuse/intestinal/mixed/not specified	-	15/16/3/9	-
Grade (differentiation)			
Well/moderate/moderate to poor/poor/not reported	-	3/8/5/29/3	-
Resectable/not resectable	-	28/15	-
Neoadjuvant (yes/no)	-	10/18	-
Adjuvant (yes/no)	-	18/10	-

^{1}H-NMR spectroscopic analyses identified and quantified 150 metabolites across all samples. After peak removal and selection for QC-RSD<25%, 77 metabolites remained. Using a critical p-value of 0.05, 28 metabolites were identified as being significantly different between GC vs. HE and 14 were significantly different between BN vs. HE; 10 of 28 metabolites were common to both groups (Table 3-2). Figure 3-1 is a biplot mapping $\log_2$ median fold change concentrations for metabolites that were significantly affected in the BN vs. HE and GC vs. HE models. Metabolites significantly affected in each pairwise comparison are listed in Table 2 in bold.

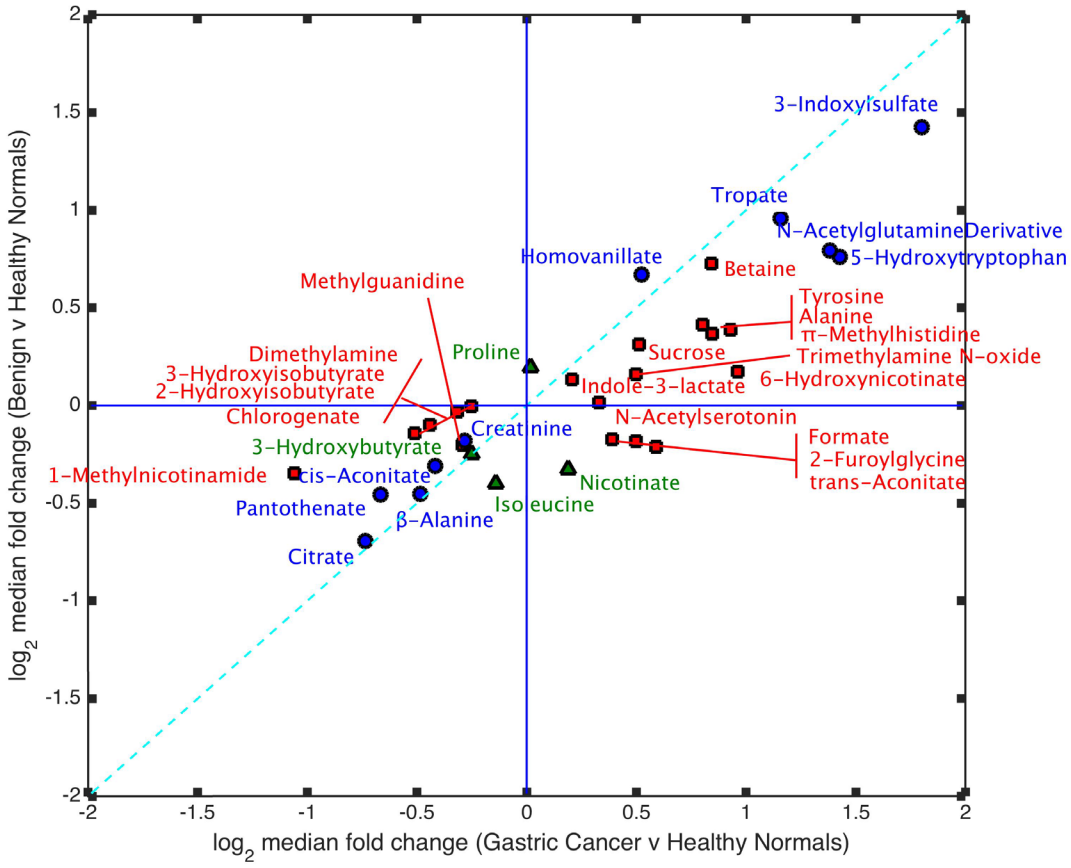


Figure 3-1. Biplot of $\log_2$ median fold change for metabolites in gastric cancer (GC) vs. healthy (HE) and benign (BN) vs. HE models. Blue circles represent metabolites significantly changed in both models; red squares, significantly changed in GC vs. HE only; green triangles, significantly changed in BN vs. HE only.

Table 3-2: Metabolic data table univariate statistics for pairwise comparisons

		Median uM			GC v HE				BN v HE			
Name	*QC-RSD*	*GC*	*BN*	*HE*	*median_fold*	*Med:diff (95% CI) uM*	*P*	*Q*	*median_fold*	*Med-diff (95% CI) uM*	*P*	*Q*
1-Methylnicotinamide	13.01	21.14	34.61	44.04	-2.08	-22.6 (-12.2 : -29.1)	**9.46E-05**	**0.00042366**	-1.27	-8.2 (4.2 : -15.1)	0.6184358	0.4888609
2-Furoylglycine	6.06	39.91	22.93	26.53	1.50	14.1 (44.1 : 0.4)	**0.01266544**	**0.02001734**	-1.16	-2.7 (8.4 : -14.5)	0.74339731	0.51453004
2-Hydroxyisobutyrate*	6.30	39.60	50.18	53.77	-1.36	-14.2 (-7.6 : -20.6)	**8.69E-06**	**7.78E-05**	-1.07	-2.9 (3.1 : -8.5)	0.24234677	0.37375076
3-Hydroxybutyrate	7.42	95.89	95.89	114.04	-1.19	-18.8 (3.6 : -61.6)	0.07705667	0.06904313	-1.19	-18 (-5.5 : -58.3)	**0.02822188**	0.12716029
3-Hydroxyisobutyrate	7.80	47.96	58.40	59.73	-1.25	-13.1 (-1.8 : -26.7)	**0.03769221**	**0.03839628**	-1.02	-1.6 (9.8 : -16.1)	0.42703806	0.44538412
3-Indoxylsulfate	21.85	204.41	157.43	58.68	3.48	143.4 (179.7 : 118.6)	**1.89E-08**	**2.55E-07**	2.68	97.3 (131.3 : 46.1)	**0.00018493**	**0.00277755**
5-Hydroxytryptophan	16.09	103.12	64.96	38.36	2.69	61.5 (93.1 : 27.9)	**0.00141075**	**0.00344738**	1.69	28.2 (73.8 : 3.9)	**0.04674937**	0.1504573
6-Hydroxynicotinate	14.98	21.08	16.69	14.93	1.41	5.5 (7.5 : 0.9)	**0.04258356**	**0.04088043**	1.12	1.1 (3.8 : -2.4)	0.86627269	0.54959245
Alanine	4.34	169.37	116.20	88.90	1.91	76.1 (117.3 : 44.6)	**8.57E-05**	**0.00042366**	1.31	25 (50.5 : -0.1)	0.18739855	0.31515702
Betaine	19.38	183.80	169.58	102.50	1.79	82.1 (134.7 : 30.7)	**0.0154339**	**0.02001734**	1.65	61.8 (113.3 : -5.4)	0.10019453	0.20355421
Chlorogenate	18.77	10.67	13.78	15.20	-1.42	-4.3 (-1.2 : -7.7)	**0.0104688**	**0.01758767**	-1.10	-1.3 (2.4 : -5.5)	0.19894584	0.3201418
Citrate	6.63	1916.93	1974.33	3193.25	-1.67	-1347.8 (-708.5 : -2362)	**1.50E-05**	**0.00010069**	-1.62	-1275 (-712.1 : -2253.1)	**0.00014418**	**0.00277755**
Creatinine	3.25	7033.34	7553.19	8561.35	-1.22	-1528 (-524.8 : -2522.8)	**0.00027171**	**0.00082544**	-1.13	-1026.7 (-360.8 : -1746.7)	**0.01413571**	0.07961468
Dimethylamine	6.05	229.07	271.94	272.70	-1.19	-36.3 (-5.1 : -67.5)	**0.02408832**	**0.02943169**	-1.00	8.1 (54.2 : -24.8)	0.44713763	0.44538412
Formate	20.54	157.60	106.72	120.35	1.31	33.1 (69.4 : 8.7)	**0.03607123**	**0.03839628**	-1.13	-14.9 (15.6 : -43.8)	0.90045027	0.54959245
Homovanillate	17.25	56.23	62.19	39.10	1.44	19 (36.9 : 2.8)	**0.01563847**	**0.02001734**	1.59	19.9 (36.9 : 3)	**0.00579505**	**0.04351822**
Indole-3-lactate	7.05	66.09	62.78	57.23	1.15	10.3 (27.4 : 4.7)	**0.03856749**	**0.03839628**	1.10	6.1 (28 : -7.9)	0.10390642	0.20355421
Isoleucine	17.72	17.52	14.64	19.32	-1.10	-2.5 (1.1 : -7.4)	0.2937666	0.16801685	-1.32	-4.9 (-0.1 : -9.9)	**0.04069973**	0.14106318
Methylguanidine	15.11	57.37	61.07	70.30	-1.23	-13 (-4.2 : -24.1)	**0.0135152**	**0.02001734**	-1.15	-9 (0.9 : -19.3)	0.06328921	0.16774784
N-AcetylglutamineDerivative	2.24	635.28	422.51	243.70	2.61	394 (467.4 : 291.8)	**9.64E-10**	**2.59E-08**	1.73	179.1 (275.4 : 120.1)	**9.58E-06**	**0.00043183**
N-Acetylserotonin	7.65	59.46	47.84	47.32	1.26	11.6 (23.1 : 2.6)	**0.00660994**	**0.01269116**	1.01	1.1 (16.2 : -10.9)	0.6948866	0.51453004
Nicotinate	23.49	9.24	6.46	8.11	1.14	0.8 (2.9 : -2.5)	0.95988369	0.3440161	-1.26	-1.7 (-0.5 : -5.2)	**0.03481483**	0.13772675
Pantothenate	8.66	23.83	24.36	33.34	-1.40	-8.9 (-5.2 : -13.8)	**0.00660637**	**0.01269116**	-1.37	-8.6 (-2.4 : -14.3)	**0.0366804**	0.13772675
Proline	6.73	214.80	242.89	212.17	1.01	14.6 (65.1 : -14.3)	0.19204547	0.13951916	1.14	37.1 (72.7 : 10.4)	**0.01240249**	0.07961468
Sucrose	21.64	183.20	159.61	128.45	1.43	57.6 (82.6 : 26.3)	**0.00040587**	**0.00109099**	1.24	29.8 (56.8 : -7.3)	0.18104008	0.31515702
Trimethylamine N-oxide	4.48	224.75	130.09	115.37	1.95	108.9 (153 : 11.8)	**0.03149249**	**0.03680532**	1.13	18 (66.4 : -39.5)	0.44145208	0.44538412
Tropate	12.43	191.18	166.60	85.72	2.23	92.9 (146.9 : 49)	**0.00027637**	**0.00082544**	1.94	77.4 (133.3 : 38.9)	**0.00354984**	**0.0355073**
Tyrosine	14.61	81.64	62.46	46.84	1.74	34 (44.8 : 14.9)	**0.00272739**	**0.00610939**	1.33	15.5 (33.4 : -2.1)	0.05428205	0.16305364
cis-Aconitate	21.61	168.12	181.02	224.39	-1.33	-64.3 (-17.2 : -119.3)	**0.00821799**	**0.01472671**	-1.24	-47.6 (-3.5 : -100.5)	**0.02558628**	0.12716029
trans-Aconitate	3.97	37.91	23.67	26.84	1.41	10.3 (19.3 : 1.5)	**0.03434724**	**0.03839628**	-1.13	-3.2 (1.7 : -7.8)	0.30788966	0.39796537
β-Alanine	16.60	112.28	129.85	178.13	-1.59	-67 (-26.7 : -98.1)	**0.00015631**	**0.00060023**	-1.37	-49.2 (-9.8 : -73.7)	**0.00394024**	**0.0355073**
π-Methylhistidine	8.19	180.54	129.64	100.54	1.80	84.4 (148.5 : 27.8)	**0.01540612**	**0.02001734**	1.29	32.4 (74.9 : -2.8)	0.09113573	0.20355421

PCA showed that there were six outlier data points and that the QC sample clustered well (Figure 3-2).

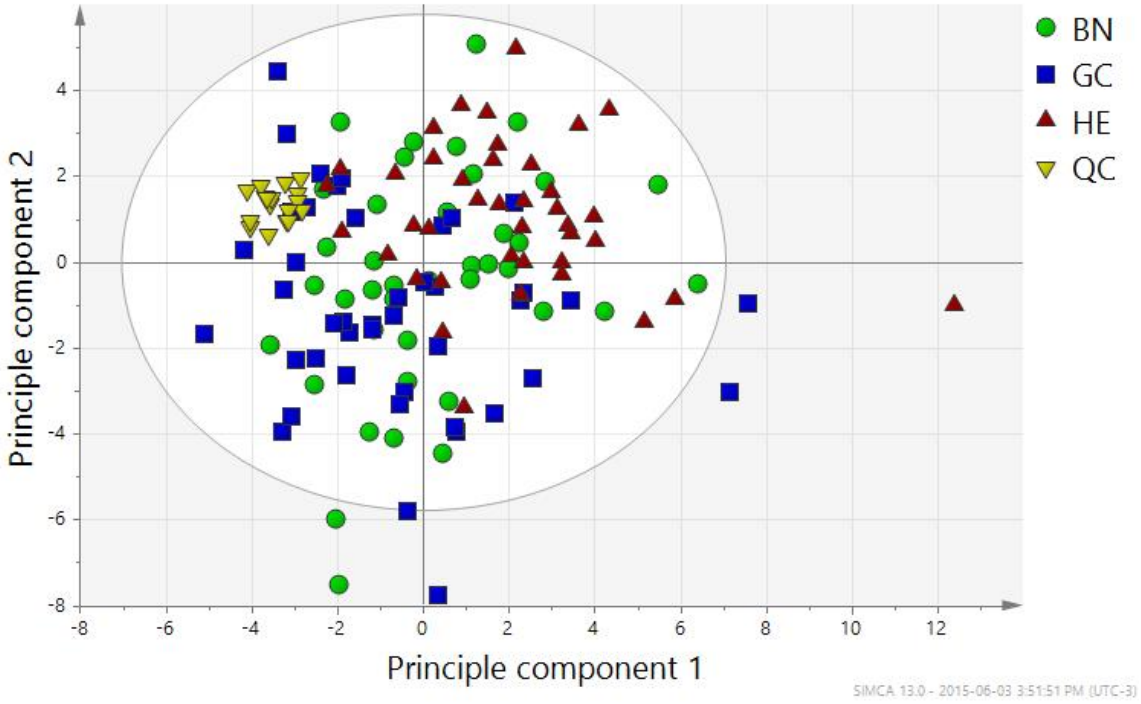


Figure 3-2. Principle component analysis (PCA) score plot of urine samples from gastric cancer ($R^2X = 0.22$, $Q^2 = 0.052$) from benign gastric disease (BN- green circles); gastric cancer (GC- blue squares); healthy controls (HE- red upright triangles) and quality control samples (QC- yellow inverted triangles).

For the compete data set PLS-DA (three disease groups) was performed. The optimal model had 1 PLS component with $R^2Y = 0.24$, $Q^2Y = 0.16$. Figure 3-3 show the PLS scores for each of the patient's metabolite profiles labeled by class.

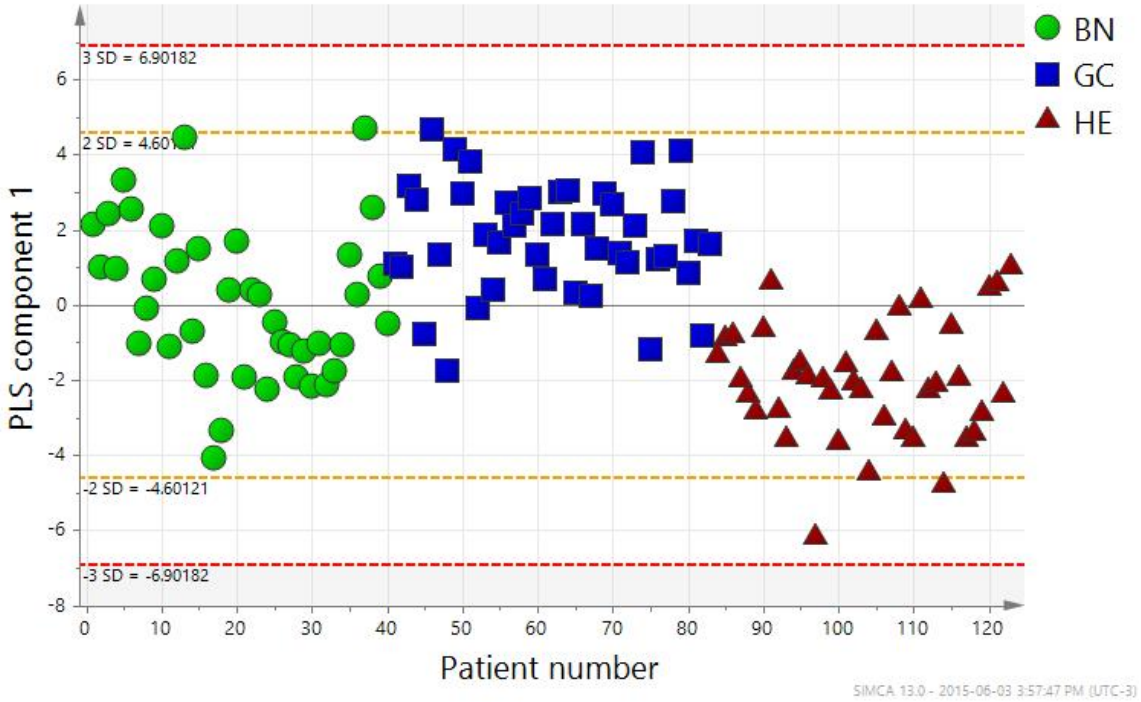
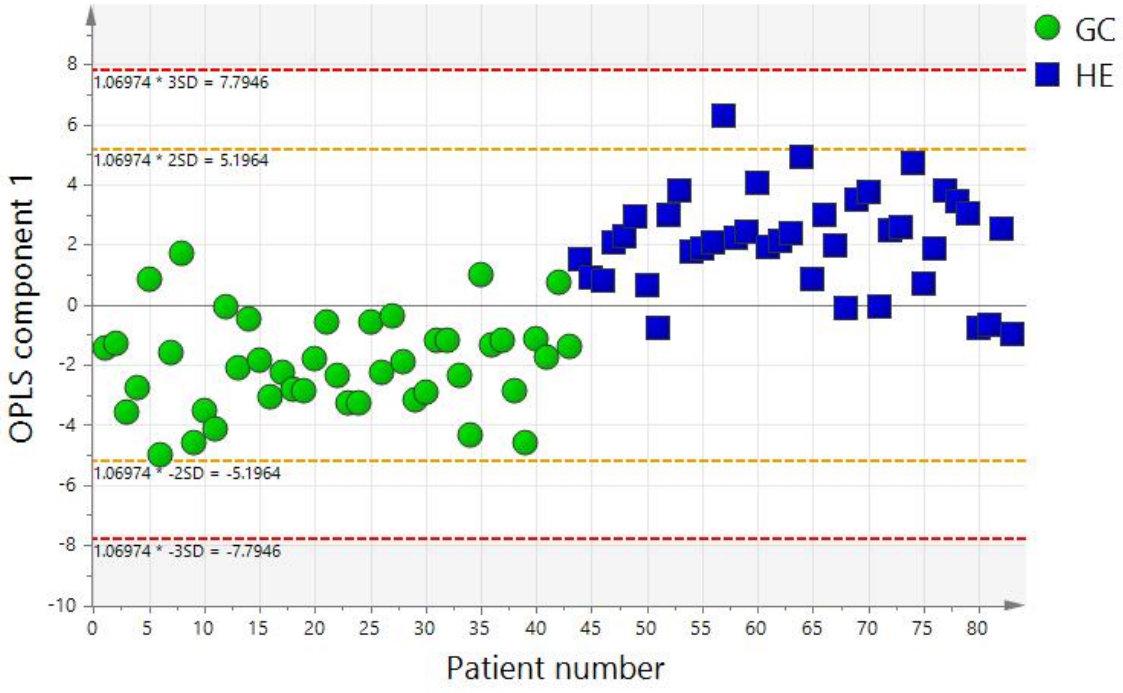


Figure 3-3. Partial Least Squares-Discriminant Analysis (PLS-DA) score plot of metabolite profiles derived from 77 measured urine metabolites in gastric cancer (GC), benign gastric disease (BN), and healthy controls (HE). Green circles are represented by BN disease patients; blue squares, GC; red triangles, HE. Cross-validated optimal model has 1 OPLS component, $R^2Y = 0.24$, $Q^2Y = 0.16$.

OPLS-DA for the GC vs. HE groups was performed (Figure 3-4) shows the OPLS scores for each of the patient's metabolite profiles labeled by class). The optimal model had 1 OPLS component with $R^2Y = 0.62$, $Q^2Y = 0.48$, and an AuROC = 0.96 (95% CI = 0.92-0.99). For a fixed specificity of 80%, the sensitivity of predicting GC was 90% (95% CI = 0.80-0.97) (Figure 3-4B shows the resulting ROC curve for GC classification).

(A)



(B)

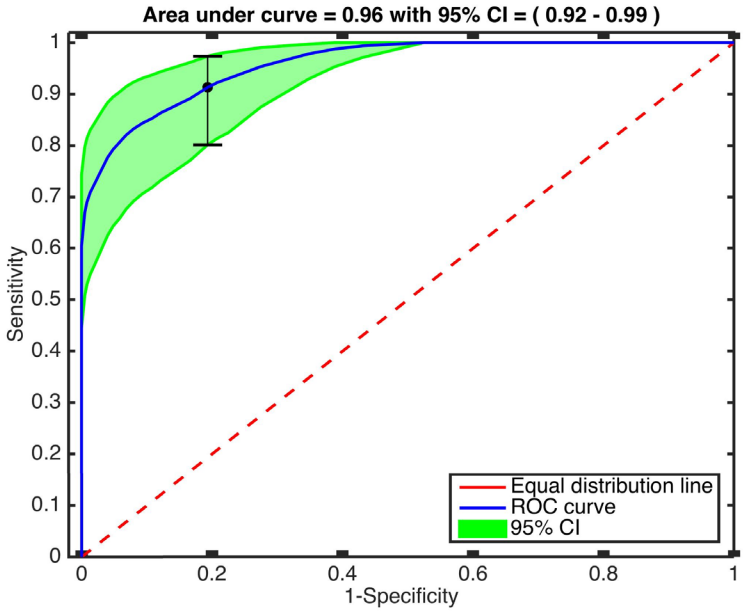
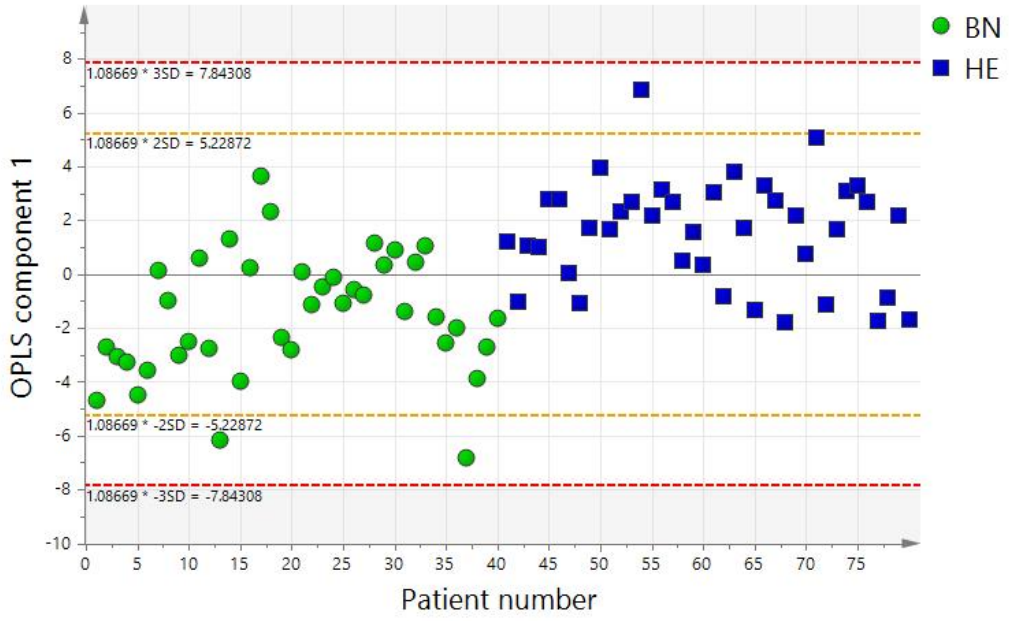


Figure 3-4.
(A) Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) score plot for GC vs HE comparison. The optimal model has 1 OPLS component with an $R^2Y = 0.62$ and $Q^2Y = 0.48$. Green circles are GC patients; blue squares, HE patients. (B) ROC curve with an AuROC of 0.96 (95% CI = 0.92–0.99). For a fixed specificity of 80% the corresponding sensitivity for predicting GC was 90% (95% CI = 0.80–0.97).

OPLS-DA for the BN vs. HE groups was performed (Figure 3-5A) show the PLS scores for each of the patient's metabolite profiles labeled by class). The optimal model had 1 OPLS component with $R^2Y = 0.35$, $Q^2Y = 0.13$ and an AuROC = 0.85 (95% CI = 0.75-0.92). For a fixed specificity of 80%, the sensitivity of predicting GC is 63% (95% CI = 0.48-0.76) (Figure 3-5B) shows the resulting ROC curve for BN classification).

(A)



(B)

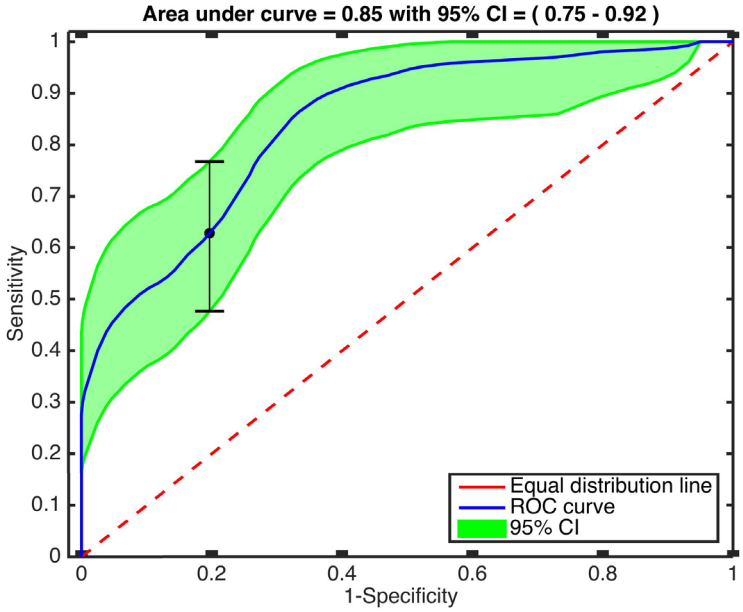


Figure 3-5.
(A) OPLS-DA score plot for BN vs HE comparison. The optimal model has 1 OPLS component with R^2Y = 0.35 and Q^2Y = 0.13. Green circles are BN patients; blue squares, HE patients.
(B) ROC curve with an AuROC of 0.85 (95% CI = 0.75–0.92). For a fixed specificity of 80% the corresponding sensitivity for predicting GC was 63% (95% CI = 0.48–0.76).

VIP scores for important metabolites in the PLS-DA and each of the two OPLS-DA models are listed in Table 3-3.

Table 3-3: VIP scores for metabolites in pairwise comparisons

	VIP score		
Metabolite	**PLS-DA GC vs BN vs HE**	**OPLS-DA GC vs HE**	**OPLS-DA BN vs HE**
3-indoxylsulfate	3.0	2.8	2.7
N-acetylglutamine derivative	2.9	3.1	2.9
2-hydroxyisobutyrate	2.1	2.3	1.0
β-alanine	2.1	1.8	1.8
Creatinine	2.0	1.9	1.8
Tropate	1.9	1.9	2.2
Sucrose	1.9	1.8	0.9
Citrate	1.8	1.9	2.6
Dimethylamine	1.6	1.4	0.5
1-methylnicotinamide	1.5	1.9	0.7
5-hydroxytryptophan	1.4	1.6	1.2
2-furoylglycine	1.3	1.4	0.3
N-acetylserotonin	1.2	1.5	0.1
Trans-aconitate	1.2	1.2	0.2
Cis-aconitate	1.2	1.2	1.5
Betaine	1.2	1.2	1.1
Alanine	1.2	1.2	0.9
Methylguanidine	1.2	1.1	1.2
π-methylhistidine	1.1	1.0	1.0
Formate	1.1	1.1	0.3
Indole-3-lactate	1.1	1.1	1.2
3-hydroxyisobutyrate	1.0	1.1	1.1
Serotonin	1.0	1.0	0.4
Tyrosine	1.0	1.0	1.0

VIP scores for metabolites in each PLS model mirrored the test scores found during the univariate statistical analysis. Of particular importance were nine metabolites, which had high VIP scores in the GC vs. HE OPLS model, but low VIP scores in the BN vs. HE OPLS model: sucrose, dimethylamine, 1-methylnicotinamide, 2-furoylglycine, N-acetylserotonin, trans-aconitate, alanine, formate, and serotonin.

OPLS-DA for the GC vs. BN groups was performed; however the model was unable to be successfully optimized, producing very poor R^2Y and Q^2Y values. Therefore it was considered inconclusive and unpublishable.

LASSO-LR produced an optimal model using just three metabolites: 2-hydroxyisobutyrate (2-HIB), 3-indoxylsulfate (3-IS), and alanine (A). This resulted in the following diagnostic regression model:

$$logit(p) = 15.0 - 7.7 \times \log(\text{2-HIB}) + 5.2 \times \log(\text{3-IS}) - 6.1 \times \log(\text{A})$$

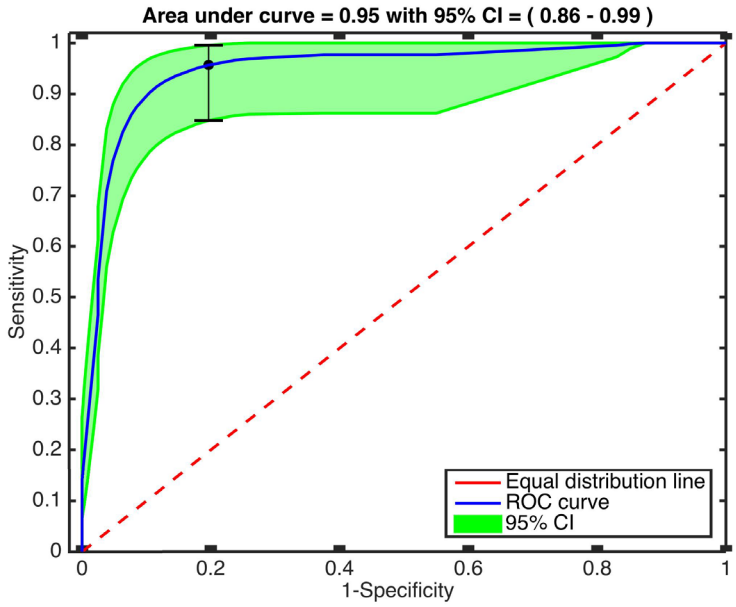
The corresponding ROC curve had an AuROC of 0.95 (95% CI: 0.86–0.99) (Figure 3-6A). For a fixed specificity of 80% the corresponding sensitivity for predicting GC was 96% (95% CI: 0.85–1.00). Model statistics are in Table 3-4. According to this specificity if the predicted score, *p,* for a given individual is > 0.3 the diagnosis would be GC; otherwise if p<0.3, "not GC".

Table 3-4: Regression parameters for Logistic Regression Model

	Estimate	SE	*t*-stat	p-value
(Intercept)	14.97	7.12	2.10	0.0355
2-Hydroxyisobutyrate	-7.77	3.01	-2.59	0.0097
3-Indoxylsulfate	5.22	1.34	3.89	0.0001
Alanine	-6.06	1.90	-3.19	0.0014

Figure 3-6B shows a frequency histogram for three disease classifications grouped by the LASSO-LR model score. BN samples are split into two broad distributions (a combined bimodal distribution); approximately half of BN patients classified with GC, the other half with HE.

(A)



(B)

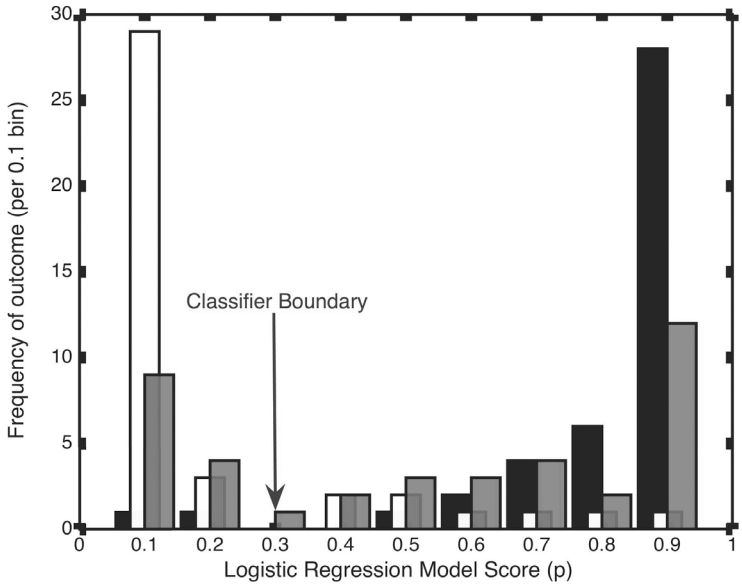


Figure 3-6.
(A) Receiver Operating Characteristic (ROC) curve for GC vs. HE comparison based on 3-metabolite model. Area under curve (AUC) is 0.95 (95% CI = 0.86 – 0.99). For a fixed specificity of 80%, the sensitivity is 95% (95% CI = 0.85-1.00).
(B) Frequency histogram for logistic regression model scores. White bars represent HE patients; grey, BN patients; black, GC patients. The number (frequency) of patients with each score is depicted by the height of the bars. Scores closer to 1 indicate a high probability of GC; close to 0 indicates high probability of HE. Cut-off boundary is score 0.3. Above 0.3, classified as GC; below, not GC

3.5 Discussion

GC is a highly morbid and fatal disease. Current screening techniques are limited, and strategies for earlier detection are necessary. Diagnosis of GC is often delayed, owing to non-specific symptoms, which also clinically overlap with symptoms of non-malignant gastric conditions such as ulcers, GERD, and gastritis. Patients may be prescribed proton pump inhibitors or antacids for presumed benign gastric aetiologies; this may mask underlying malignancy symptoms. The present study used 1D ^{1}H-NMR spectroscopy to characterize a urinary metabolic profile of GC that is distinct from HE and a subpopulation of BN patients.

Univariate analysis revealed significant changes to 28 metabolites measured in urine of patients with either GC or BN gastric disease when compared to healthy matched controls (Table 3-2); these included carbohydrates, vitamins, organic and amino acid metabolites. The biplot in Figure 3-1 showed log fold change in concentration for significant metabolites in the GC vs. HE and BN vs. HE models. Some interesting metabolites unique to the GC phenotype are: alanine, 1-methylnicotinamide, sucrose, and methylguanidine.

Alanine is an endogenous amino acid that can be synthesized from pyruvate, or 3-phosphoglycerate, a glycolytic intermediate. Five to seven percent of skeletal muscle is composed of alanine.[24] During times of fasting, muscle protein is catabolized to release alanine as a substrate for liver gluconeogenesis. Alanine provides the main carbon skeleton for gluconeogenesis, which produces glucose as an energy source for cancer cells. Like previous studies,[15, 25] alanine concentration increased from HE to GC, and was also significantly correlated with tumour stage.[26]

A lung cancer study showed that compared to healthy controls and weight-stable lung patients, weight-loss lung patients upregulated gluconeogenesis and had higher alanine concentrations.[27] Several GC patients had lost a considerable amount of weight from baseline to diagnosis of cancer, and elevated alanine levels in urine may be an indication of increased lean muscle breakdown.

Both 1-methylnicotinamide and sucrose have connections with gastric mucosal inflammation. In rats with chemically induced gastric lesions (ulcers, erosions), treatment with 1-methylnicotinamide inhibited gastric acid secretion, and increased mucosal blood flow and healing. These changes were mediated through induction of gastro-protective prostaglandins.[28] Diminished levels of 1-methylnicotinamide in both BN and GC groups suggest loss of this mucosal protective mechanism, and may correlate with gastric mucosal damage observed in these patients.

Elevated urinary sucrose levels may be related to gastric mucosal integrity. In the 1990s, Meddings devised the sucrose permeability test, based on the premise that healthy gastric mucosa is intact and does not allow sucrose to leak excessively into the bloodstream.[29] However, in areas of ulceration or erosion, sucrose can penetrate more easily into the bloodstream and be excreted into the urine. After oral administration of a sucrose load, urinary sucrose levels showed steady increases going from healthy patients to those with gastric ulcer, early GC, and finally advanced GC.[30, 31] Our results agreed with previous studies, as there were significant sucrose elevations in both BN and GC groups compared to baseline.

Perhaps this is due to the increased permeability of damaged mucosa in GC and BN patients.

Some interesting metabolites common to both models in the biplot are: creatinine, citrate, and cis-aconitate and 3-indoxylsulfate. Creatinine, a waste product of muscle metabolism, is excreted by the kidneys.[32] The amount of creatinine in urine is directly related to muscle mass.[33] Cancer cachexia patients have lower total body skeletal muscle mass, and therefore lower levels of creatinine in urine. This phenomenon was consistent with our results as GC patients had considerably lower levels of urinary creatinine than HE patients. In this experiment only BMI was measured; a formal body composition analysis was not completed, but several GC patients reported muscular atrophy in the months preceding initiation of malignancy treatment.

Citrate is an intermediate of the Kreb's cycle: complete oxidation of this metabolite provides a major source of cellular ATP. It is also an important regulatory metabolite at the junction of several interconnected biochemical pathways- fatty acid and sterol biosynthesis, glycolysis and gluconeogenesis.[34] With regard to cancer, an *in-vitro* experiment showed that citrate induced apoptosis in two GC cell lines in a dose dependent manner.[10] In our study citrate was down-regulated in GC patients, suggesting an ability of GC to escape regular programmed cell death. Dehydration of citric acid produces cis-aconitate, one of the metabolites identified in this experiment.[35]

Finally, 3-indoxylsulfate is a metabolite of the amino acid tryptophan[36] and a nephrotoxin that stimulates glomerular sclerosis and interstitial fibrosis.[37] A

Korean research group found that 3-indoxylsulfate was nearly tripled in mice injected with GC cells;[17] this mirrored our findings as GC concentrations of this metabolite were approximately 3.5 times that of controls. It was also increased in oesophageal cancer;[38] 3-indoxylsulfate is postulated to increase oxidative stress by decreasing levels of glutathione, a cellular anti-oxidant.

OPLS modeling clearly distinguished GC metabolomic profiles from those of HE (Figure 3-4) and to a lesser extent BN from HE (Figure 3-5). Identification of unique and important GC phenotype metabolites was consistent between multivariate and univariate methods. Nine metabolites have VIP >1 in GC/HE comparison but VIP <1 in BN/HE comparison. Eight metabolites contributing to the GC phenotype with VIP >1 matched significant metabolites identified on univariate analysis: sucrose, dimethylamine, 1-methylnicotinamide, 2-furoylglycine, N-acetylserotonin, trans-aconitate, alanine, and formate.

The distinction between BN and either GC or HE was less clear using the multiclass PLS model (Figure 3-3). Overall, alanine, 3-hydroxyisobutyrate and 2-indoxylsulfate are most predictive of GC. Interestingly, due to the phenotypic heterogeneity of the BN group, a bimodal distribution of patients was observed, as approximately half clustered with HE and the other half with GC.

BN conditions that clustered more frequently with HE include: varices, polyps, portal hypertensive gastropathy, gastroparesis, and gastritis. BN conditions that clustered more frequently with GC include: ulcers, GERD, and gastritis. These observations fit with Correa's hypothesis.[39] He delineated a pre-neoplastic cascade from healthy to non-atrophic/superficial gastritis to chronic atrophic gastritis (CAG)

to intestinal metaplasia (IM) to dysplasia (DYS) to cancer. Patients with chronic gastritis are farther on the pre-neoplastic cascade than early gastritis patients, so their phenotypes and metabolomic signature more likely resemble GC than they do HE. Likewise patients with mild superficial gastritis have minimal inflammation and are more similar to HE.

While results of this biomarker discovery experiment have offered additional insight into GC, a number of limitations in the design, methods, and analysis stages should be addressed. In the design phase, there are issues of sample size/power and confounding variables. As the strength of relationships between urine NMR profiles and the differentiation of metabolomic profiles between benign and malignant disease is currently unknown, sample size could not be formally calculated. Therefore, we enrolled a pragmatic sample size of 40 GC patients, 40 patients with gastrointestinal symptoms and without GC (BN group), and 40 healthy controls. A small sample size limits the power to detect a difference, and likewise, differences detected may be spurious.

This experiment matched patients on three common confounders- age, sex and BMI, but as it is an observational design, all known and unknown confounders cannot be controlled. Randomization can better achieve this prognostic balance, but as samples were not randomly selected, only run order was randomized. Since the experiment was completed over several days, randomization minimized any spurious batch effects. Other confounders in this experiment include: patient medications, smoking, *Helicobacter pylori* status, and non-uniform endoscopy between groups. Medication profiles amongst patients can be quite heterogenous;

moreover interactions of multiple drugs on the metabolome are difficult to predict. While all BN and GC patients underwent upper endoscopy for symptoms, HE patients did not. It is still possible that this group may have stomach pathology that is clinically occult. Some GC patients were not fasting at the time of urine collection, which could influence the metabolomic profile. BN patients were all fasting as that was mandatory for scoping, as were HE patients.

In the analysis phase, limitations pertain to the ^{1}H-NMR spectrometer and the Chenomx library. The ability to detect metabolites is restricted by the sensitivity and resolution of the instrument, as well as the number of identified compounds in the Chenomx library. The machine used in this experiment was exceptionally precise from run-to-run, but NMR can only detect metabolites with a concentration ≥1 μm;[40] furthermore distinguishing overlapping/co-resonant spectral peaks can make accurate identification of individual metabolites difficult. The latest version of the Chenomx software houses about 340 known compounds, but there were several peaks, some of which were significant between GC vs. HE, that were unknown and not available in the library. These were removed from further analysis.

In the future, there can be improvements to the design, methods and analysis stages. Both the original biomarker discovery and an external validation experiment should employ a sufficiently large sample size (at least as large as the current experiment) to detect a difference in metabolomic profiles. A larger external validation set will be important in identifying whether the same metabolites emerge as discriminatory biomarkers of disease. Patient samples can be randomly collected, although in Canada, where the incidence of GC is only 2-3%,

this may be difficult. It could take a long time to accrue enough GC patients, unless multiple centers pool their patients. A validated standard operating procedure should be applied uniformly to all patients. Everyone should be fasting and the time of day at which samples are collected should be similar. Ideally patients should be enrolled, and samples collected and analyzed within a tight time frame to minimize time and temperature dependent alterations in biochemical profile. Any HE patients who participate should also be scoped within the last six months to ensure absence of stomach pathology.

A sufficiently powered longitudinal study can examine the metabolomic profile in GC patients post-operatively at six week and six month intervals. The same standard operating procedure should be applied to post-operative samples. After curative resection, some metabolites may return to normal levels. One study found that as early as seven days after surgery, alanine, arginine, and hypoxanthine trended towards healthy concentrations.[26]

The National Cancer Institute (NCI) outlined 30 steps required to move "omics" assays from bench to bedside.[41] Once a validated standardized protocol for specimen collection/storage and experimental assay is identified, then there are multiple steps to developing a valid biomarker model. Thereafter an "omics" assay enters the clinical trial design stage after which multiple ethical, legal and regulatory issues must be resolved before it can be considered for a clinical test. Once a test is of sufficient quality for clinical use, a risk stratification model should be constructed to identify the best candidates for the urine-screening test. Like the Gail model in breast cancer,[42] patients with different combinations of risk factors for GC can be

triaged to have this urine test. If a patient's metabolomic profile is classified with GC, then he or she can be further worked up with endoscopy and/or barium swallow. Such a test serves as starting point for investigation, and enables clinicians to correctly target a small group of patients in a more efficient and cost effective manner.

3.6 Conclusion

In this observational cohort study we demonstrated that GC has a unique urinary metabolomic profile compared to HE patients, and a subset of BN patients (mostly ulcers and chronic gastritis). A parsimonious three metabolite model consisting of 2-hydroxyisobutyrate, 3-indoxylsulfate, and alanine was established that predicted GC as distinct from HE with 95% sensitivity and 80% specificity. This study shows the clinical potential for urinary metabolomic profiling in GC patients, although numerous steps are required to first validate these findings, and then to advance it to a clinically applicable test.

Acknowledgements

We would like to thank the following individuals and organizations for their contributions to this manuscript:

- Laboratory technicians Michelle Kuzma and Delores Mowles for assistance with adjusting the pH and preparation of urine samples for NMR
- Medical secretary, Rose Cornand, for assistance with patient consent and urine collection at the Royal Alexandra Hospital

- Gastroenterologists (Dr. Clarence Wong), surgeons (Drs. Klaus Buttenschoen, Cliff Sample, Erika Haase, Gordon Lees) and their respective administrative staff for referring study patients
- Endoscopy suite staff at Royal Alexandra and University of Alberta Hospitals- use of their facilities to recruit patients
- Patients for donating urine samples to make this project possible
- Research financial support- Edmonton Civic Employees Charitable Assistance Fund, Alberta Innovates Technology Futures Graduate Student Scholarship, Dr. D. Schiller Academic Enrichment Fund, Queen Elizabeth II Graduate Scholarship
- Dr. Dean Eurich holds a Canada Research Chair from Government of Canada and Alberta Heritage Foundation for Medical Research (AHFMR) Population Health Investigator Award from Alberta Innovates Health Solutions (AIHS).

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CHAPTER 4: SUMMARY

4.1 Summary of Research

4.1.1 Overview

Metabolomics is the "study of the quantitative complement of metabolites in a biological system and changes in metabolite concentrations or fluxes related to genetic or environmental perturbations".[1] Metabolites are small molecules (<1500 Da) in a biological organism and are the breakdown products of multiple convergent gene, protein and biochemical pathways. Since the 1990s, research in this field has grown exponentially. Metabolomics has important applications in nutritional science, drug metabolism, and increasingly now in the area of oncology diagnostics and therapeutics.[2] Malignancies of the pancreas,[3] breast,[4] esophagus,[5] kidney[6, 7] and lung[8, 9] have been studied previously, but very little has been done yet in the area of gastric cancer (GC).

GC is a devastating disease and the average mortality rate (all stages combined) is approximately 70%. Most GC cases occur in East Asia. Despite only 3300 cases of GC in Canada, and 250 cases in Alberta every year, the mortality rates of 62% and 66% respectively are nearly on par with the rest of the world.[10, 11] Much of the morbidity and mortality is related to late diagnosis as symptoms of GC often overlap with those of benign (BN) disease. As the prevalence of GC is low in Canada and screening would not be cost effective, there is no official screening program for this malignancy. Given the high mortality of the disease, and the limitations to

current screening modalities, such as endoscopy and barium swallow, tests to identify GC earlier are highly valuable.

4.1.2 Current Evidence

Chapter 2 of this thesis summarized the research on GC as of 2014. Multiple studies report disruptions to carbohydrate metabolism in GC cases. The transition from healthy to malignant phenotype is correlated with an upregulation of lactate and a downregulation of glucose, consistent with the postulated Warburg Effect.[12-15] Cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than aerobic oxidation of pyruvate in mitochondria as in normal cells. Malignant cells have very high rates of glycolysis and therefore glucose consumption. Overall, this adaptation has enabled cancer cells to survive in relatively hypoxic environments. Amino acid, lipid, and nucleic acid metabolism also show disruptions, but they are considerably less consistent between literature articles.

Many studies featured a case-control design, but to date, no study has looked at three disease groups (GC, BN, and healthy controls- HE) with a large enough sample size. While it is interesting to see differences between healthy controls and cancerous cases, it is also important and perhaps more clinically useful to identify whether metabolomic profiles differ between cancer and benign disease. It is precisely these two groups of patients who are presenting the diagnostic dilemma, as healthy asymptomatic individuals will not seek medical attention. An improved understanding of metabolic biomarkers and their functional significance may

provide an opportunity for early identification and treatment of GC. Therefore Chapter 3 sought to identify whether there was a difference in urinary metabolomic signature between three disease groups (GC, BN and HE) using ^{1}H-NMR spectroscopy.

4.1.3 Disease Class Separation

Using univariate and multivariate statistical analysis, we found that GC has a discrete metabolomic signature compared to HE and a subset of BN patients, namely chronic gastritis and ulcers. On univariate analysis, 28 metabolites initially differed between GC and HE patients, of which 10 also differed between BN and HE patients. There is strong agreement between univariate and multivariate results as metabolites that were significant discriminators of cancer on Mann-Whitney U test were virtually the same as metabolites with high variable importance in projection (VIP) scores on partial least squares-discriminant analysis (PLS-DA). A discrete metabolomic profile was not identified for the BN group of patients, as the parameters for the multivariate GC vs BN model could not be successfully optimized. This is likely due to phenotypic heterogeneity of the BN disease group as a whole.

LASSO Logistic Regression was used to investigate the predictive ability of combining multiple metabolites in a single model. Three compounds were selected as the key discriminatory biomarkers (2-hydroxyisobutyrate, 3-indoxylsulfate, and alanine). This parsimonious triple metabolite biomarker model shows strong predictive accuracy in separating disease classes, with an area under the receiver operating characteristic curve [(AuROC) of 0.95, (95% CI =0.86-0.99)]. This three

metabolite assay can be used as an initial diagnostic test to screen for high risk patients. Any individuals with a Logistic Regression model score above 0.3 (our cut point for the test) can be further investigated for possible stomach cancer with other tests such as endoscopy. Given that endoscopy is a timely, and resource intensive service, any method to identify a smaller, more appropriate population for this test can be more practical, cost effective and efficient. This relatively non-invasive and economical urine metabolic screen may serve this purpose.

4.1.4 Metabolites of Interest

Knowledge of cancer hallmarks[16] may allow researchers to speculate upon the functional significance of the metabolites identified in this experiment. There are multiple explanations for the role of a particular metabolite in cancer, as individual metabolites participate in multiple biochemical reactions. The products of one reaction may be the intermediates or substrates of another. The putative biomarkers identified in this discovery experiment provide a basis for future in-depth studies of gastric carcinogenesis pathways.

This research identified a number of potential metabolites postulated to be involved in gastric tumor formation and propagation. Metabolites of muscle breakdown, mucosal damage, and cellular proliferation were identified. Creatinine and alanine, an endogenous amino acid, were prominent biomarkers identified and are both implicated in muscle catabolism. Alanine can be released from skeletal muscle, and shuttled to the liver for gluconeogenesis.[17] Creatinine, a waste product of muscle metabolism, is correlated with total body muscle mass.[18, 19] Although a

formal body composition analysis was not undertaken, many cancer patients reported significant weight loss, muscle atrophy, and had lower urinary creatinine levels. Cancer cachexia occurs in 50% of patients with malignancy,[20] and has been considered a "paraneoplastic syndrome" in which tumour derived factors induce global alterations in gene expression and metabolic flux to release metabolites which can then be channeled to other pathways for tumour growth and expansion.[21]

GC grows from the mucosa towards the serosa of the stomach. Damage to the mucosal lining is correlated with alterations in 1-methylnicotinamide and sucrose concentrations. A previous study shows that 1-methylnicotinamide enables gastric mucosal healing in rats who have ulcers and erosions. This metabolite was downregulated in GC patients in our experiment, indicating loss of this mucosal protective mechanism. Sucrose, on the other hand, has not been linked to a particular dysregulated biological pathway but leaks into the bloodstream and subsequently the urine in higher quantities when the mucosa is damaged compared to when it is intact.

Other amino acids such as isoleucine, proline, and tyrosine were significantly different between cancers and controls as well, and this may reflect a need for the tumour to increase protein synthesis. Proteins are essential constituents of cell membranes and signaling molecules.

The pattern of metabolic derangements associated with GC is speculative and explanations for biological significance are limited by the literature that is available on this topic. Further in-depth analysis of these metabolites may be undertaken with Ingenuity Systems Pathway Analysis,[22] an online application which allows

researchers to navigate molecular relationships between candidate biomarkers. Combining multiple levels of "omics" technologies may help to generate a more global and comprehensive picture of GC genesis and propagation at the gene, mRNA and metabolite levels.

4.2 Limitations and Future Directions

4.2.1 Sample Size and Power

While results of this biomarker discovery experiment have offered additional insight into GC, a number of limitations in the design, methods, and analysis stages should be addressed. In the design phase, one of the issues is sample size and power. Sample size could not be formally calculated because the strength of relationships between urine NMR profiles and the differentiation of metabolomic profiles between benign and malignant disease is currently unknown. No published guidelines exist either on an appropriate sample size. Previous GC metabolomics studies used anywhere from 8 to 65 patients per group.[12, 14] Therefore, we enrolled a pragmatic sample size of 40 GC patients, 40 patients with gastrointestinal symptoms and without GC (BN group), and 40 healthy controls.

A small sample size limits the power to detect a difference, and conversely, differences detected may be spurious. In "omics" experiments, the number of variables (metabolites) greatly exceeds the number of samples. In the search for disease discriminatory biomarkers, multiple hypotheses are being tested simultaneously for each metabolite. This increases the chance of making false discoveries (Type I error).[23] For example, if a univariate test sets the significance

level at p-value 0.05, there is a one in twenty chance that the biomarker is false, but if one were to perform >100 tests (as is the case of this metabolomics experiment), then the chance of finding a false biomarker is greatly amplified. The more tests that are performed, the greater the chance of finding a random metabolite that is not biologically relevant.[1]

Different correction methods for multiple hypotheses testing have been utilized. The Bonferroni correction controls the family-wise error rate by dividing the overall desired p-value by the total number of hypotheses performed. For example, if the desired significance level is 0.05 overall and there are 100 tests being performed, then the significance level for each individual test is now 0.0005. This correction method can be overly stringent, as it can avoid Type I errors at the cost of increasing the potential for Type II errors (missing a true association).[23] Bonferroni correction also assumes that all variables are independent of one another, which in reality, may only apply to a small number of metabolites in each sample.[22] We utilized the Benjamini-Hochberg method to correct for multiple hypotheses. This is a calculation that ranks p-values to produce a new adjusted q-value. Ultimately, this method serves to reduce the number of false discoveries.[25, 26]

4.2.2 Bias and Confounding

This experiment matched patients on three common confounders- age, sex and BMI, but as it is an observational design, all known and unknown confounders cannot be controlled. The heterogeneity of human characteristics can make it virtually impossible to control for all confounders. Since patients cannot be

randomized to a disease group, only experimental run order was randomized to minimize any spurious batch effects.

Other potential confounders in this experiment include: patient medications, smoking, *Helicobacter pylori* status, and non-uniform endoscopy between groups. Medication profiles amongst patients can be quite heterogenous; moreover the interaction of multiple drugs on the metabolome is difficult to predict. While all BN and GC patients underwent upper endoscopy for symptoms, the HE patients did not. It is still possible that this group may have stomach pathology that is clinically occult, which could lead to misclassification. For example, if a healthy individual has mild gastritis without symptoms, he or she may be misclassified into the control group, rather than in the benign gastric disease group.

With regard to the experimental methods, there were important differences in sample collection and storage time that could affect metabolomic profile. Some GC urine samples were collected years before initiation of the current experiment. Although they were stored in -80 °C, subtle changes to metabolites may occur even at this temperature. The oldest GC samples from 2009 were biobanked for nearly four years before they were thawed for experimental analysis. This is in contrast to the BN and HE patients who were collected within a tight four-month time frame from August to November 2013. No article has been officially published regarding the optimum storage time and temperature, but the consensus in the metabolomic community is that beyond 1.5 years, even in -80 °C, time dependent degradation processes such as proteolysis and lipidolysis occur. Therefore age of samples can influence the validity of identified metabolomic profiles.

Another difference between cancer and non-cancer samples is the fasting state of patients. Some of the GC patients that were collected were not fasting at the time of urine collection, which could influence the metabolomic profile. A large proportion of GC patients in the biobank were collected prior to the initiation of this MSc program of research in 2013, so fasting was not a necessary pre-requisite. In contrast, the BN patients were all fasting as that was mandatory for scoping. Likewise, HE patients were fasting as they were all collected under the same protocol by the same researcher.

There needs to be a validated standard operating procedure applied uniformly to all patients in the study to ensure reproducibility. Everyone should be fasting and the time of day at which samples are collected should be similar. Ideally patients should be enrolled, and samples collected and analyzed within a tight time frame (two year window if possible) to minimize time and temperature dependent alterations in biochemical profile.

4.2.3 Biofluids and Analytical Platforms

In the analysis phase, limitations pertain to the ^{1}H-NMR spectrometer and the Chenomx software library. The ability to detect metabolites is restricted by the sensitivity and resolution of the instrument, as well as the number of identified compounds in the Chenomx library. The NMR device used in this experiment was exceptionally precise from run-to-run, but the lower limit of detection for NMR is 1 μm concentration;[28] any metabolite below this level is not detectable. Furthermore distinguishing overlapping/co-resonant spectral peaks can make accurate

identification of individual metabolites difficult. The latest version of Chenomx (version 7.7) has about 340 known compounds. There were several peaks, some of which were significant between GC and HE that were unknown and not available in the library. These had to be removed from further analysis.

Metabolomics can use a variety of fluid or tissue samples, most commonly urine or serum is utilized. We chose to use urine because it is sterile, easy to collect, painless, is reasonably free from interfering proteins or lipids, and requires minimal processing for NMR. However, urine is not without its challenges. Urine typically contains water-soluble metabolic breakdown products of diet, environmental contaminants, endogenous waste metabolites and bacterial by-products. It is a final common channel for multiple sources of breakdown products, many of which are not well characterized or understood.[28] Every compound that is found in urine should theoretically also be found in blood, albeit in different concentrations. Due to the filtration of substances from blood into the kidneys, the urine metabolome should be a subset of the serum metabolome. However, according to the Human Metabolomic Database (HMDB), there are nearly 500 compounds that are identified in urine that are not detected in blood. This may be due to the concentrating ability of the kidney for certain metabolites. Some compounds that are too low concentration to be detected in blood can be found in higher concentrations in urine.[28] No one particular biofluid or analytical platform can tell the entire story of a patient's metabolome, so different biofluids and platforms should be combined to provide complementary analyses of the human metabolome.

4.2.4 Implications for Future Research

In the future, there can be improvements to the design, methods and analysis stages. Both the original biomarker discovery and an external validation experiment should employ a sufficiently large sample size (at least as large as the current experiment) to detect a difference in metabolomic profiles. An external validation set will be important in identifying whether the same metabolites emerge as candidate biomarkers of disease. The external validation set should consist of all new individuals. Each specimen should be collected under an identical and reproducible standard operating protocol to reduce bias and confounding. This time, healthy individuals who participate should also be scoped within the last six months so that no occult stomach pathology is identified that could confound the metabolomic profile. An external validation set can first be collected in the same hospitals in the Edmonton area, and then be repeated in other centers. If further validation studies identify a similar metabolomic profile to this experiment, then this lends more strength to the validity of these metabolites as biomarkers of GC.

Beyond looking for differences in metabolomic profile between disease groups like in this current research program, other ideas for future experiments include investigating the effects of surgical resection and cancer recurrence on the metabolomic profile. A sufficiently powered longitudinal study can examine the metabolomic profile in GC patients post-operatively at six week and six month intervals. The same standard operating procedure should be applied to post-operative samples. After curative resection, do key discriminatory metabolites return to normal levels? Do post-operative patients still cluster with cancer patients

on multivariate analysis, or are their metabolomic profiles more consistent with healthy individuals? One study found that as early as seven days after surgery, alanine, arginine, and hypoxanthine trended towards healthy concentrations.[29] Likewise, the metabolomic signature can also be studied in patients with recurrent GC to identify whether the same metabolites of malignancy return. In this way, each patient can be his or her own control at different time points.

Another idea for a future experiment could investigate the effects of *Helicobacter pylori* bacteria on the overall metabolomic profile. It would be interesting to identify which metabolites are of bacterial versus human metabolic origin. In this current experiment, not all patients' *H. pylori* infection status were known, as information was missing from health records.

It is hoped that these candidate metabolites may emerge as reasonably robust biomarkers for disease detection in future validation experiments. Ultimately they may serve some purpose as a diagnostic screening tool. However, there are still major challenges to moving "omics" experiments from bench to bedside. McShane and colleagues discussed 30 steps required to transition a biomarker discovery experiment to a population level clinical tool.[30] Apart from what has already been mentioned in terms of specimen and collection assay standardization, further considerations include model development, specification, and evaluation of statistical methods. Then the "omics" based test must pass clinical trials, and finally overcome numerous ethical, legal and regulatory issues.

An ideal screening program should have the right balance of disease, test, and population characteristics.[31] Pertaining to the disease, early detection and

therefore intervention of the condition in question should have a significant effect on quality of life. The prevalence of the disease should be high enough to justify health economic costs. There should be effective treatment for early stage disease and a long enough asymptomatic period during which detection and treatment reduces morbidity and mortality. It takes on average 44 months for early stage GC to progress to advanced stage; during this transition period, patients are often asymptomatic.[32] With regard to the characteristics of the test, it must be sufficiently sensitive enough to detect disease during the asymptomatic period, specific enough to minimize false positives, and acceptable to patients (painless, minimally invasive, few risks or side effects). A urine metabolomics screening test has several favourable patient factors already. Finally, the population characteristics must also be considered. The disease should be sufficiently prevalent, and patients should have reasonable access to medical care. There should be enough compliance with subsequent diagnostic tests and therapy. In East Asia, GC is much more common, and such a screening program is justifiable given that several conditions of a good screening test are met. In Canada on the other hand, stomach cancer is relatively rare (2-3% prevalence);[10] however, there are certain subpopulations (East Asian immigrants and First Nations)[33] who are at higher risk for GC and could potentially be a target group for this urine screening test in the future.

These characteristics must operate in conjunction with an appropriate risk stratification model. A strategy to identify the correct target population for the screening test is important. Knowledge of previous risk factors can be used to build a risk stratification tool. Like the Gail model in breast cancer,[34] patients with

different combinations of risk factors for GC can be triaged to have this urine test. A positive screen can fast track them towards further work-up with other tests.

This experiment has shown compelling results for metabolic profiling in the diagnosis of GC. Additional large-scale studies and integration of current knowledge with information from other "omics" studies (proteomics, genomics, etc) may foster a better understanding of the biological processes underpinning gastric adenocarcinomas.

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