

**Nutrigenetic and Metabolomic Approaches to Study the Role of Dietary
Antioxidants and Plant Bioactives in Human Health**

By

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ABSTRACT

Clinical, epidemiological and interventional research evidence suggests that low-grade chronic inflammation is strongly linked to inflammatory disease conditions including inflammatory bowel disease (IBD), inflammatory bowel syndrome, diabetes mellitus type 2, insulin resistance, and hypertension. By far, the most successful prevention/ treatment strategy in such conditions is an anti-inflammatory lifestyle, including diet, physical activity, and medications. A substantial amount of evidence supports that foods, nutrients and non-nutrient components can modulate inflammation, therefore contributing to overall health, leading to better health outcomes. Dietary antioxidants originate primarily from plants. Some are essential nutrients such as ascorbic acid, while many others are bioactive compounds classified as non-nutrients, including the polyphenolic compounds commonly abundant in fresh fruits and vegetables.

There is much research on the relationship between dietary antioxidants and inflammatory diseases. However, the dietary recommendations designed to manage inflammatory conditions such as IBD, insulin resistance and type 2 diabetes mellitus are often based on inconclusive or contradictory findings and sometimes on non-existing evidence. Therefore, the objective was to gain a better understanding of this relationship using novel approaches in two well-designed research studies. The first study entitled “Nutrigenetics of ascorbic acid and IBD” was aimed at the characterization of a potential ascorbic acid transporter, and revealed the genetic associations of ascorbic acid transporters with IBD. In the results section of this thesis (starting with chapter 4), an orphan gene “sodium-dependent ascorbic acid solute carrier 23 gene: *SLC23A3*” was characterized for the first time in terms of genomic location, size, alternative splicing patterns, protein isoforms structure prediction, tissues expression profiles, and association of its genetic

variants with IBD. Data sourced from gene databases were analyzed utilizing a bioinformatics approach, and gene expression was performed using real-time quantitative polymerase chain reaction (RT qPCR). DNA samples of inflammatory bowel disease (IBD) patients were genotyped for SNPs on the *SLC23A3* using Taqman® PCR. The findings showed that *SLC23A3* is located on 2q35, *SLC23A3* (8.63 kb in size) and exhibits three major splice variants encoding for 3 isoforms that vary in their transmembrane domains. Various tissues expressed substantial levels of *SLC23A3*, including renal, hepatic, and intestinal epithelial tissues. Although *SLC23A3* was highly expressed in the intestinal tissues, none of the genetic variants of choice was associated with IBD. The second part of the same study (chapter 5) investigated the genetic association of the sodium-dependent ascorbic acid transporter gene *SLC23A2* with IBD. Genomic DNA samples of 288 Crohn's disease (CD) cases, 265 ulcerative colitis (UC) cases and 269 matching controls were genotyped (4 SNPs) using RT PCR, followed by logistic regression analysis. rs33560557 variant (GA carrier) and (AA carrier) increased susceptibility to UC by 3.4 and 3.49-folds ($P < 0.01$), respectively. GA genotype increased susceptibility to CD by 2.93 folds ($P < 0.01$). Variant rs33560557 may have a functional role in maintaining plasma levels of ascorbic acid in IBD patients. In the second study, "Metabolic responses to Saskatoon berry yogurt" (chapter 6), a functional food "Saskatoon berry yogurt (SBY)" was developed, and examined for its functionality in a group of healthy participants, in terms of glucose, insulin, and antioxidant responses. A randomized, controlled, cross-over trial was conducted with 12 healthy participants aged 18-35 yrs. Participants were given SBY, yogurt and bread. Plasma concentrations of glucose and insulin were measured at fasting and 15, 30, 60, 90, and 120 minutes post-meal consumption. Glucose and insulin incremental area under the curve from 0 to 120 minutes were calculated. SBY antioxidant capacity was measured, and its plasma total

antioxidant capacity response was measured at 0, 45, 90 and 180 min post-ingestion. Phenolic compounds and their metabolites were detected in Saskatoon berry powder (SBP), SBY, plasma, and urine. Ingesting SBY, a low glycemic index (low GI) food, showed lower glucose response after 60 and 90 minutes than white bread ($P < 0.05$) and higher antioxidant responses than plain yogurt. This was most likely attributable to the SBP phenolic content. Drawing on these findings, SBY is a potential low GI functional food, replete with antioxidants from SBP. Findings from both studies are meant to narrow the gap between the extensive research base on the relationship between dietary antioxidants and inflammatory diseases and the lack of conclusive, personalized dietary guidelines.

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FORWARD

The manuscript for the first study “chapter 4” was presented as a poster at the Canadian Nutrition Society’s conference (2014), and is being prepared for publication in *Genes*. The manuscript of the second study “chapter 5” is being prepared for publication in *The American Journal of Clinical Nutrition*. The third manuscript “chapter 6” was presented as a poster at the DREAM Diabetes Research Symposium (2016), and is being prepared for publication in *British Journal of Nutrition*.

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ABBREVIATIONS

AceView	Acembly View
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CHO	Carbohydrate
CI	Confidence interval
CNPPD1	Cyclin Pas1/ PHO80 domain-containing protein 1
CV%	Coefficient of variation percent
D ₂ O	Deuterated water
dbSNP	Single nucleotide polymorphism database
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENSEMBL	Ensembl genome database project
EPIC	European prospective investigation into cancer and nutrition
EST	Expressed sequence tag
FAM134A	Family with sequence similarity 134 member A
FRAP	Ferric reducing antioxidant power
GEO	Gene expression omnibus repository

GI	Glycemic index
GL	Glycemic load
GLUT2	Glucose transporter 2
GRCh38	Genome Reference Consortium Human build 38
¹ H NMR	Proton nuclear magnetic resonance
HbA1c	Glycated hemoglobin
iAUC	Incremental area under the curve
IBD	Inflammatory bowel disease
IHH	Indian hedgehog signaling molecule
INF- γ	Interferon gamma
LC-QTOF-MS	Liquid chromatography quadrupole time of flight mass spectrometry
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MHQ	MassHunter quantitative analysis software®
MHz	Megahertz
MIR3131	microRNA 3131
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NCBI	National center for biotechnology information
ND	Not determined
NHEJ1	Non-homologous end joining factor 1
NMR	Nuclear magnetic resonance
OR	Odds ratio

ORF	Open reading frame
pH	Potential hydrogen
PN	Personalized nutrition
PubChem	Database of chemical molecules
RAW 264.7	Macrophage-like Abelson murine leukemia virus transformed cell line
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SB	Saskatoon berry
SBP	Saskatoon berry powder
SBY	Saskatoon berry yogurt
SBP/DBP	Systolic blood pressure over diastolic blood pressure
SD	Standard deviation
SEM	Standard error of mean
SPSS	Statistical package for the social sciences
sFPKM	Significant Fragments Per Kilobase of exon per Million fragments mapped
SLC23	Solute carrier family 23
SLC23A2	Sodium dependent ascorbic acid transporter 2
<i>SLC23A2</i>	Sodium dependent ascorbic acid transporter 2 gene
SLC23A3	Solute carrier transporter 3
<i>SLC23A3</i>	Solute carrier transporter 3 gene
SNP	Single nucleotide polymorphism
REB	Research ethics board
RT PCR	Real time polymerase chain reaction

RT qPCR	Real time quantitative polymerase chain reaction
TAC	Total antioxidant capacity
TMHMM	Prediction of transmembrane helices in proteins
TNF	Tumor necrosis factor
3' UTR	3' untranslated region
5' UTR	5' untranslated region

Chapter 1: General Introduction

1.1 Introduction

Given that maintaining overall homeostatic balance is important to health and well-being, it is important that nutrients and their metabolites reach their target tissues. Additionally, it is critical that they also be functionally active at the cellular and the molecular levels (Hediger et al, 2004). Diet is a major exogenous factor to our overall health, contributing to the many different molecular mechanisms during health and disease states. Multiple chemicals derived from our foods originate from plant sources, and of these, plant antioxidants are of particular interest.

The prevalence of metabolic diseases, especially those of inflammatory origin, or conditions associated with low-grade inflammation, is on the rise globally. The latter includes cardiovascular diseases, cancer and inflammatory bowel disease (IBD) (Rosella et al, 2015; Viennois et al, 2016). Dietary compounds that possess antioxidant activity act against the generation of excess free radicals that can cause cellular damage resulting in diseased states. Even when the damage has occurred, antioxidants have the potential to alleviate the progression of oxidative damage by preventing further damage to cells (Seifried et al, 2007).

Ascorbic acid is the primary systemic non-enzymatic exogenous antioxidant (May, 1999), with many inflammatory diseases linked to an altered ascorbic acid status (Michels et al, 2013). As such, ascorbic acid transporters and the genes encoding them are integral in ascorbic acid homeostasis (Brüzle et al, 2012). Several genetic variations are known to affect ascorbic acid status directly (Michels et al, 2013), while others predispose individuals to intestinal inflammation (Lees et al, 2011). Of special interest is the solute carrier family 23 genes (ascorbic acid transporter genes): *SLC23A1*, *SLC23A2* and *SLC23A3*. Recently, a variation in the *SLC23A1* was tested for susceptibility to inflammatory bowel disease and found to predispose individuals

to Crohn's disease (Shaghghi et al, 2014). However, the potential roles of the other family members, namely *SLC23A2* and *SLC23A3*, are yet to be examined.

Phenolic compounds, which are abundant in seasonal fruits like Saskatoon berries (SB), are non-nutrient compounds that possess strong antioxidant activity (Dudonné et al, 2015; Lavola et al, 2012). These compounds were proven to be anti-inflammatory and capable of improving insulin sensitivity according to evidence provided from *in vitro* experiments and animal studies (Hu et al, 2005; Juríková et al, 2013). Additionally, human glycemic responses were positively modulated after ingestion of a mixture of berries with a phenolic profile comparable to SB (Edirisinghe et al, 2016). The health benefits of SB phenolic compounds have been demonstrated in *in vitro* experiments (Hu et al, 2005; Juríková et al, 2013). Moreover, the anti-inflammatory effect of Saskatoon berry powder (SBP) has been examined in an *in vivo* study using a diabetic mouse model (Zhao et al, 2014). Despite a great deal of evidence supporting the beneficial health effects of fruit polyphenols, there is still insufficient knowledge related to the cause and effect relationships with respect to human studies.

Given that, many interactions occur between the diet and the metabolome, the analyses of metabolites is inherently complex. On the other hand, recent advancements in analytical chemistry techniques, including the use of high-resolution quadrupole time of flight based mass spectrometry and nuclear magnetic resonance techniques, have made the study of particular food components, along with their potential effects on human health more convenient and reliable. As such, metabolomics research approaches may yield novel biomarkers to explore causal relationships between dietary components and metabolic responses.

There is a continuous need for the availability of novel functional foods year round that as both appealing and safe options for the human consumer. Functional foods containing berries

have the potential to protect against further oxidative damage as well as enhance the glycemic control in pre-diabetics or diabetics. SB is a promising ingredient not commonly used in foods and can be best delivered through low glycemic index foods such as yogurt. A comprehensive and detailed assessment of the chemical composition and the effects of SB on various metabolic responses in a sample of healthy participants in preparation for its use as a functional food is warranted.

There is a consensus that the metabolic response to particular dietary components varies widely among individuals of the same age, sex and overall health status (Elnenaei et al, 2011; Hanhineva et al, 2015; Velzen et al, 2014). Traditionally, the average response to a dietary intervention may be predicted for a group of individuals. However, the exact metabolic response(s) within each individual may vary dramatically and is influenced by their genetic variation (Elnenaei et al, 2011). Recent advances in nutrigenetics have led to the development of better tools to elucidate the nutrition-gene link in order to optimize health through the identification of novel biomarkers. These tools provide powerful explanations that describe the complex link between genetic variations, particularly single nucleotide polymorphisms (SNPs), nutrients and whole system responses. These tools will enhance our knowledge of the mechanisms leading to personalized diagnosis, dietary management and treatment modalities.

Despite the availability of research designs that combine personalized diagnosis and dietary intervention trials into practice, recommendations based on personalized nutrition efforts are focused on individuals with metabolic disorders and not on those with genetic disorders. Personalized nutrition recommendations based on research findings have been utilized only in a few conditions. Familial hypercholesterolemia, hypolactasia, and infant inborn errors of metabolism (eg. phenylketonuria, maple syrup urine disease) are a few such examples. More

research directed towards identifying and testing the effect of less common SNPs (biomarkers) is needed (San-Cristobal et al, 2013). This is particularly true for genes related to the functional regulation (homeostasis) of oxidative stress, inflammation and metabolic pathways and new environmental factors affecting the SNPs under consideration.

With respect to the structure of the current thesis: 1) Chapter 2 provides a brief review of the recent literature; 2) Chapter 3 lists the hypotheses and objectives of the two primary studies; 3) Chapters 4 and 5 will discuss findings from the first study; 4) Chapter 6 will discuss findings from the second study, in detail, findings from both studies will address the relationship between dietary antioxidants and health outcomes; and finally, 5) Chapter 7 provides a general discussion, conclusion, limitations of both of the studies, and recommendations for future directions.

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Chapter 2: A Review of the Literature

The relationship between diet and human health is complex. Understanding the link between dietary components, their metabolism, and effects on human health warrants a deeper investigation at the molecular level. Further study of the cellular pathways involved in oxidative balance, inflammatory responses, and the genetics controlling and influencing these processes is also needed. Additionally, the study of the metabolic products of these pathways and their corresponding physiological effects is necessary. In the following sections of this chapter, an overview of the relevant literature will be presented. The focus will be presented based on the most recently published scientific evidence.

2.1 Antioxidants and free radicals

The primary action of antioxidants is to neutralize free radicals and their actions (Devasagayam et al, 2004). They act as catalysts in biochemical reactions to counteract the oxygen free radicals that are being generated (Kuhn et al, 2003). Antioxidants neutralize free radicals or their actions/ products by different processes. Ascorbic acid, vitamin E, carotenoids, flavonoids, and polyphenols perform their actions by scavenging free radicals (Devasagayam et al, 2004).

Free radicals are chemical stressors (Bjorklund and Chirumbolo, 2017) generated through biochemical processes at the cellular level and play a role in maintaining homeostatic balance (Devasagayam et al, 2003; Kuhn et al, 2004). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), the main cellular stressors, are generated by both mitochondrial and endoplasmic reticulum metabolic processes. These include but are not limited to adenosine triphosphate (ATP) production and the synthesis of prostaglandins and leukotrienes. With respect

to ROS, such as superoxide and hydrogen peroxide ions, they are generated regularly in almost all healthy cells to maintain homeostatic balance and serve as signalling molecules. Despite their relatively short half-life (milliseconds), they are implicated in the etiology of many complex diseases including inflammatory metabolic impairments (Bjorklund and Chirumbolo, 2017; Devasagayam et al, 2004). The link between ROS and inflammatory diseases is explained by elevated oxidative stress. Under normal conditions, ROS are generated and regularly kept in check by the various antioxidant mechanisms in the body. However, when the balance between ROS and antioxidants is shifted towards ROS production, as a result of various environmental and pathological agents, oxidative stress occurs (Devasagayam et al, 2004).

2.2 Dietary antioxidants

Cells are equipped with endogenous enzymatic and non-enzymatic antioxidant protective mechanisms against free radical damage. Endogenous enzymatic mechanisms include glutathione reductase, glutathione peroxidase, and superoxide dismutase. Exogenous antioxidants obtained via the diet include essential nutrients such as vitamin A, C and E, minerals/trace elements and other non-essential bioactive compounds including carotenoids, polyphenols, lipoic acid and glutathione (Devasagayam et al, 2004; Kuhn et al, 2003). Most of the dietary antioxidants consumed are derived from plants. Plant species grown in harsh conditions are especially rich with phenolic compounds. These compounds act as antioxidants against multiple sources of oxidative stress. Edible phenolic-rich plants serve as exogenous antioxidant sources and, if consumed regularly, they will promote health (Bjorklund and Chirumbolo, 2017; Rodriguez-Mateos et al, 2014).

Plants synthesize a wide range of structurally and functionally diverse secondary metabolites including polyphenolic compounds and flavonoids. These compounds can be found in very high concentrations in a variety of plant species. These metabolites protect the plant from herbivores, infections, ultraviolet radiation, and may play a role in attracting pollen-dispersing animals (Rodriguez-Mateos et al, 2014). In humans, flavonoids and polyphenols (collectively known as plant-derived phytochemicals) can promote health by activating cellular pathways that lead to an optimal stress response. One potential mechanism whereby phytochemicals may act is via serving as xenobiotics for the cells triggering a stress that can act as a signal for the cellular stress response (Bjorklund and Chirumbolo, 2017). This will result in an optimal response to the original stressor and, subsequently, lessen damage. Vitamin C and SB polyphenols are of special interest in this research.

2.3 Ascorbic acid (vitamin C)

Ascorbic acid (vitamin C) is an essential nutrient which acts as a water soluble electron donor and a co-factor in enzymatic reactions. Ascorbic acid is ubiquitous in body tissues (fluids and cells), and is the most abundant hydrophilic antioxidant (Carr and Lykkesfeldt, 2018; Ellulu, 2017; Michels et al, 2013). As a potent antioxidant nutrient, it protects metabolically active cells from oxidative damage by the enzymatic and non-enzymatic mediated removal of reactive oxygen species (Arrigoni and Tullio, 2002), and therefore, plays a role in disease prevention (Ellulue, 2017). Due to its abundance in various body tissues, altered levels of ascorbic acid can affect various functions, and may result in many different disease states. Low plasma ascorbic acid associates with overall immunity, inflammation, hypertension, obesity, diabetes and others (Carr and Maggini, 2017; Ellulu, 2017). On the other hand, a dose of the vitamin (1 or more

folds the recommended dietary allowance) reduces risk of depression associated with shift work, improves pulmonary function and myocardial injury in smokers, improved mood and reduces neural damage (Ellulu, 2017).

Ascorbic acid-rich dietary sources are fresh fruits and vegetables, but also includes foods fortified with ascorbic acid (ie: fruit juices) and nutritional supplements (Mangels et al, 1993). An average healthy adult would need 75 (females) and 90 (males) mg/day. In half a cup of strawberries, 50% of the RDA is covered. 1 medium sized red bell pepper provides more than 100% of the RDA (Leong and Oey, 2012; Sapei and Hwa, 2014).

2.3.1 Absorption, transport and regulation

The total pool of ascorbic acid pool is dispersed between the plasma and tissues, with particular enrichment in the brain and neurons. Maintaining adequate levels of ascorbic acid in the body is crucial for optimum health. A severe deficiency of ascorbic acid results in the fatal disease scurvy. Epidemiological and clinical research findings indicated that hypovitaminosis C (plasma level of $<23 \mu\text{mol/L}$) can result in an increased chronic disease risk, including blood glucose and lipid abnormalities (Carr and Maggini, 2017; Ellulu, 2017). In order to maintain an overall ascorbic acid homeostatic balance, dietary and/or supplemental ascorbic acid must be absorbed across the intestinal epithelia. This absorption will result in increased plasma levels in advance of tissue uptake (Figure 2.1). At the level of the kidney, glomerular filtration withdraws some of the circulating ascorbic acid in preparation for reabsorption. As a result, plasma levels are reduced below normal levels and require replenishment. To replenish ascorbic acid, two regulatory mechanisms work to bring the balance back and restore plasma levels. These include ascorbic acid tissue efflux, as well as renal reabsorption (Michels et al, 2013). Regulation of

ascorbic acid homeostasis is controlled by several factors, including active transport mechanisms. The latter is mediated by the sodium-dependent ascorbic acid transporters SLC23A1 and SLC23A2. Two major sites for this regulation include intestinal absorption and renal reabsorption (Figure 2.2), controlled primarily by the ascorbic acid active transporter SLC23A1. SLC23A1 mediates ascorbic acid influx into enterocytes and renal epithelial cells across the apical side (at the brush-border of intestinal and renal lumina) (Michels et al, 2013; Washko et al, 1993; Wilson et al, 2005). It is believed that ascorbic acid has different physiological fates once inside cells. The generally assumed important fate for homeostatic balance maintenance is the efflux of ascorbic acid across the basolateral side via an active transporter, (Eck et al, 2013; May and Qu, 2009) yet to be identified.

Figure 2.1: Ascorbic acid regulation mediated by SLC23A1 and SLC23A2*

At the intestinal lumen, dietary ascorbic acid is absorbed thereby increasing plasma levels. Subsequently, almost every cell in the body absorbs circulating ascorbic acid. Simultaneously, at the kidney, glomerular filtration withdraws some of the circulating ascorbic acid in preparation for reabsorption. When plasma ascorbic acid levels go down, tissue efflux along with renal reabsorption work to effect a balance. At several points, ascorbic acid transporters regulate this balance. However, the full set of transporters has yet to be determined. SLC23A3 is positioned as a putative ascorbic acid transporter

**Figure 2.1 adapted from (Michels et al, 2013)*

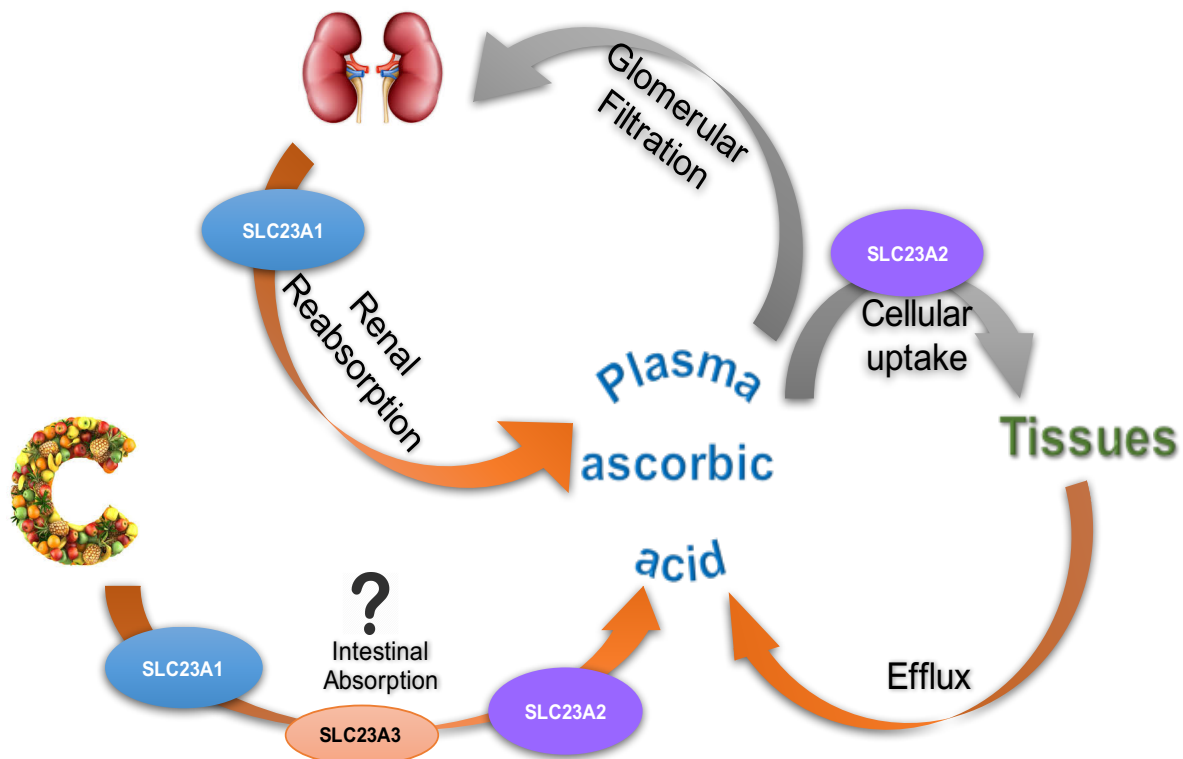
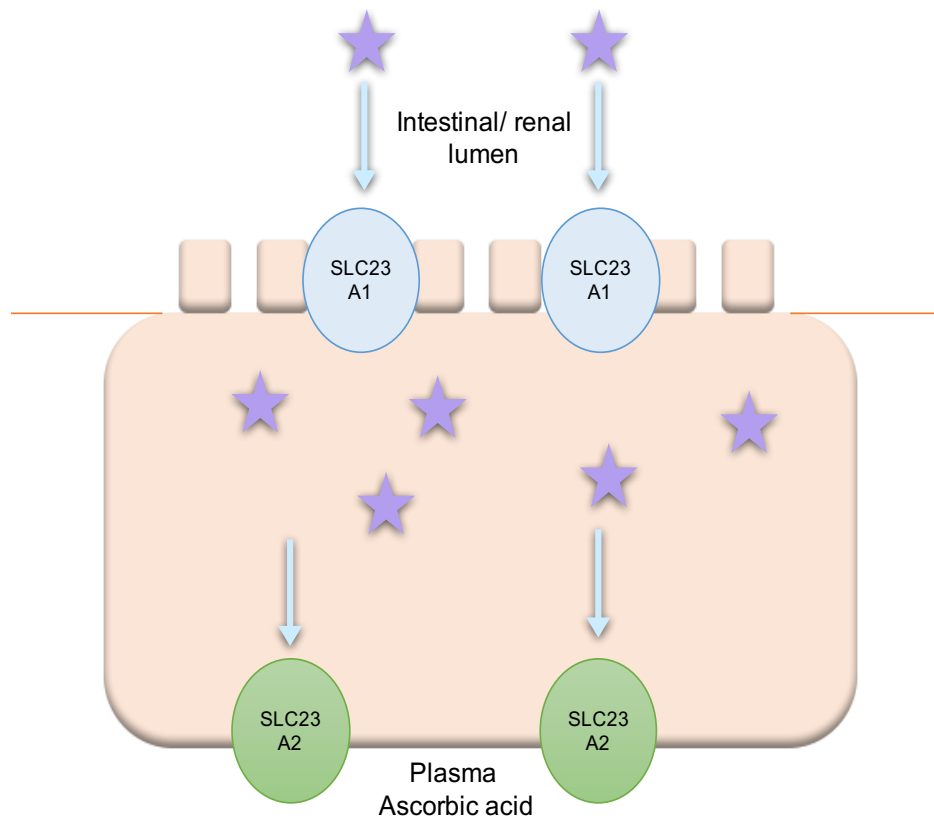


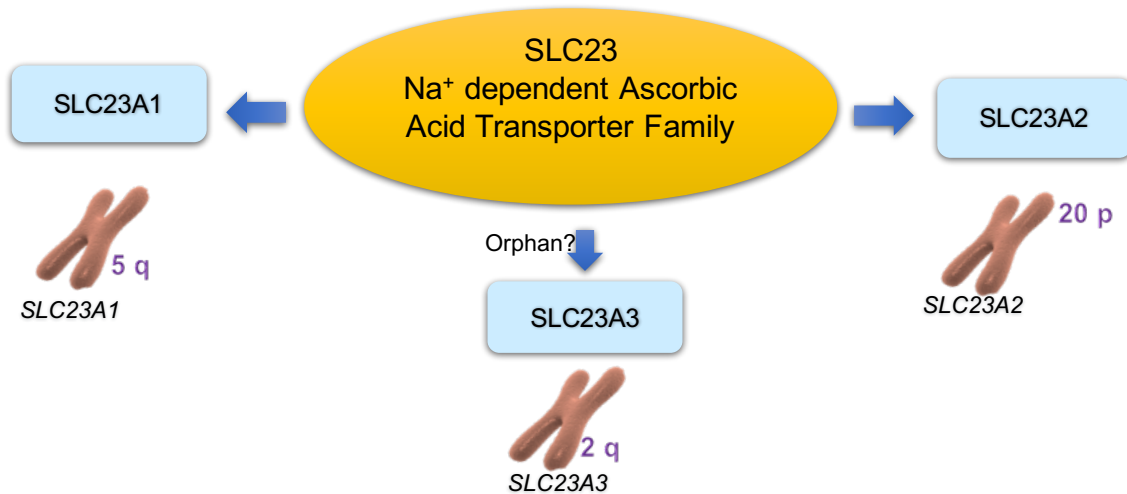
Figure 2.2: SLC23A1 and SLC23A2 mediate ascorbic acid absorption and reabsorption
Ascorbic acid (purple stars) intestinal absorption and renal reabsorption at the apical and the basal sides of the intestinal and renal epithelia are mediated by SLC23A1 and SLC23A2, respectively



2.3.2 The sodium-dependent ascorbic acid transporter gene family SLC23

In order for body cells to maintain an overall homeostatic balance, the absorbed nutrients and their metabolites must cross the epithelia, and reach their target tissues. In so doing, nutrients and their metabolites will have the potential to be functionally active at the molecular level. Several transport systems exist in humans to deliver the various nutrients, metabolites, and drugs throughout the body. Solute carriers are comprised of several families of transmembrane transporters, with each having the capacity to move substances across cellular and subcellular membranes (Hediger et al, 2004; Vasiliou and Nebert, 2008). Solute carrier family 23 (SLC23) is a subset family of the solute carrier superfamily. Existing in all vertebrates, SLC23 is composed of 2 well-characterized sodium dependent ascorbic acid transporters SLC23A1 and SLC23A2, as well as an “Orphan” member SLC23A3 (Figure 2.3) (Daruwala et al, 1999).

Figure 2.3: The sodium-dependent ascorbic acid transporter gene family SLC23.
The SLC23 consists of 2 sodium dependent ascorbic acid transporter proteins namely: SLC23A1, SLC23A2 and an orphan member (SLC23A3). Genes encoding for these transporters sit on different parts of the chromosome. SLC23A1 and SLC23A2 are well characterized (both genes and proteins), but the SLC23A3 has yet to be characterized.



2.4 Phenolic compounds

There is an increased interest in plant phenolic compounds and their respective role(s) in maintaining cellular antioxidant balance. The involvement of dietary polyphenols and their metabolites has become an important field of study, especially for human nutrition research aimed at health promotion. Phenolic compounds and their metabolites are synthesized via the shikimate pathway or during phenylpropanoid metabolism. They are naturally abundant in plant species as they play an important role in self defense against different stressors such as ultraviolet radiation and herbivores, as well as antibacterial, antiviral and antifungal agents. Phenolic compounds also participate in various cellular repair mechanisms. Furthermore, when consumed, they contribute to the organoleptic properties to the plants, both taste and colour. Once ingested, phenolic compounds exert many beneficial physiological effects at the molecular and cellular levels (Rodreguez-Mateos et al, 2014; Tarko et al, 2013).

Collectively, phenolic compounds can be divided into 3 main groups. Based on their chemical structure, one group includes the flavonoids and related phenolic acids, phenolic compounds and polyphenols. Another second group constitutes the terpenoids and nitrogen-containing alkaloids. Finally, the third group consists of the sulphur-containing compounds (Rodreguez-Mateos et al, 2014; Tarko et al, 2013).

2.4.1 Absorption, distribution, metabolism and excretion

To better understand the physiological effects of phenolic compounds on human health, it is necessary to review absorption, distribution, metabolism and excretion of ingested polyphenols. Dietary polyphenols happen to occur as glycosides, esters and polymers. As such, these molecules will not be absorbed until they are hydrolyzed by digestive enzymes or gut

microflora. Following ingestion, salivary enzymes (such as beta glucosidase) will modify the structure in the oral cavity by hydrolyzing polyphenol glycosides. Hydrolyzed polyphenols will pass through the stomach mainly unchanged, en route to the distal intestines. Recent findings estimated that 10% of the ingested dietary polyphenols remain undigested in the food particles, with 48% digested in the small intestine and 42% digested in the large intestine. Once in the small intestine, a cascade of enzymatic reactions will take place, followed by absorption at the duodenum. All undigested polyphenols, upon reaching the colon, will be further degraded into smaller phenolic acids through the action of colonic microflora. This will be followed by bioconversions and ultimately absorption into the main circulation. Depending on the specific polyphenol and its derivatives, metabolites and site of digestion, urinary excretion of phenolic compounds will usually take place within 2-24 hours (Rodreguez-Mateos et al, 2014; Tarko et al, 2013).

2.4.2 Berries as sources of phenolic compounds

Wild berries are considered an ideal source to study the phenolic compounds and their effects on human health due to their antioxidant properties. Wild fruits are not influenced by commercial breeding, fertilization and genetic modification (Kraft et al, 2008). In North America, berries are native to Western Canadian culture, and these include the Saskatoon berries (*Amelanchier alnifolia* Nutt.), or (Saskatoons).

2.4.3 Health benefits of phenolic compounds of berries

The role(s) of secondary metabolites in human metabolism is becoming increasingly important. Accumulating evidence has demonstrated that the long term intake of plant polyphenols (fruits and vegetables) can positively affect the incidence of chronic diseases, including cancers, diabetes mellitus type 2, cardiovascular diseases, and impaired cognitive function (Rio et al, 2013).

Berries have been shown to have anti-diabetic and anti-inflammatory properties, and to decrease lipid oxidation biomarkers, as demonstrated in *in vitro* experiments. (Hu et al, 2005; Dudonné et al, 2015). Saskatoon berry extract prevented cellular oxidation induced by peroxy radical in mouse macrophage cells (Hu et al, 2005). Anthocyanins, a major group of water-soluble phenolic pigments, have been reported to be the bioactive compounds responsible for this protective effect of berries against the metabolic diseases caused by oxidative stress (Dudonné et al, 2015; Juríková et al, 2013). The anthocyanins of fruit extracts have also been shown to impair nitric oxide in LPS/INF- γ -activated RAW 264.7 macrophages (Wang and Mazza, 2002). Furthermore, *in vitro* and *in vivo* evidence indicates that phenolic compounds in berries exhibit a variety of therapeutic effects. These include inhibition of low density lipoprotein (LDL) oxidation, vasodilation, apoptosis induction as well as anti-carcinogenic, antidiabetic, antiulcer and antitumor activities (Vuong et al, 2005). Saskatoon berries have been shown to possess antioxidant activity according to evidence from *in vitro* experiments (Hu et al, 2005). Several studies have explored the phytochemical profiles of different types of berries, but only focused on a specific group of compounds.

2.4.4 Saskatoon berry uses and chemical composition

Historically, the usage of Saskatoon berries dates back to the traditions of the Indigenous people of North America. The Indigenous people of Canada used sun-dried and smoked Saskatoon berries for flavour. Fresh and dried fruits were used in a variety of food preparations and dishes commonly consumed as soups, stews, meat dishes, pemmican and dried cakes (Mazza, 2008). European settlers benefited from these berries, and were able to use them throughout all the seasons in Western Canada as fresh, baked in pies, or processed into jams (Bakowska-Barczak et al, 2007; Mazza, 2008).

The chemical composition of Saskatoon berries has not been extensively analyzed to date. The basic composition of the fruits consists of water as a major constituent with an average of 83%, followed by 15-20% carbohydrates (mainly sugars), and to a lesser extent, small amounts of fat and protein. According to the same reports, Saskatoon berries are good sources of fibers, potassium, iron, manganese and phosphorus. The vitamin content was found to include vitamins A, B₁, B₂, B₅, B₆, folate, C, and E (Mazza, 2008). In addition to its nutrient profile, there is a good content of phenolic compounds. Many reports indicate that Saskatoon berries are a very good source of a variety of bioactive phenolic compounds (Dudonné et al, 2015; Lavola et al, 2012). Anthocyanins are the major functional components (Mazza, 2008), followed by flavonols, procyanidins, and phenolic acids (Lavola et al, 2012). Quantitatively, a range of 25 to 179 mg of anthocyanins/100 grams of berries have been reported (Mazza, 2008). A recent report showed the anthocyanin content was as much as 382 mg/100 g of fresh weight making it the richest source among the cultivated berries (Lavola et al, 2012). Cyanidin 3-galactoside and cyanidin 3-glucoside are the two main compounds accounting for approximately 61% and 21% of total anthocyanins, respectively (Mazza, 2008).

Several factors in the field and present during harvesting, storage and processing affect the phenolic content of fruits. Cultivar, maturity level, season and year of production, storage conditions, and drying are among the factors that have a great impact on the anthocyanin and the total phenolic contents of Saskatoon berries (Mazza and Cottrell, 2008; Mitra et al, 2013). Freeze-drying is one of the best preservation methods for retaining the phenolic profiles, and the quality of the fruits. A recent study found that freeze-dried Saskatoon berries retained greater amounts and activity of its antioxidants, as observed by the DPPH and ABTS radicals scavenging activity, comparing to other drying methods (Mitra et al, 2013). The recent development of innovative food processing methods has enhanced the opportunities for using Saskatoon berries in the food industry. Examples of innovative food products containing processed Saskatoon berries include cereals, ice creams, juices and multiple snack foods (Bakowska-Barczak et al, 2007; Mazza and Cottrell, 2008).

2.4.5 Saskatoon berry health benefits

The indigenous peoples of North America used Saskatoon berries for various health benefits. The fruit juice was mainly used to treat stomach disease while the mature fruits were used as ear and eye drops (Mazza and Cottrell, 2008). Because of its scientifically proven health benefits, the interest in the cultivation of Saskatoon berries has increased over the past few years. Cultivars have been commercially distributed to Europe (for example Finland) and some Asian countries such as Japan. This expansion has been possible by the improved climate and harvesting environments (Bakowska-Barczak et al, 2008). Given the observed health benefits from *in vitro* studies, and the lack of studies conducted on humans in this regard, Saskatoon berries are the target food to be investigated in the current study. With respect to *in vivo*

evidence, studies with diabetic mouse models have clearly demonstrated the potential therapeutic benefits from Saskatoon berries. Two reports using a diabetic mouse model (db/db diabetic dyslipidemic mice), successfully tested the protective and therapeutic effects and the underlying mechanisms of SBP. The first study, which incorporated 5% freeze-dried SBP in the regular diet, concluded that SBP or its bioactive compounds, especially cyanidin glycols, attenuated the endoplasmic reticulum stress induced by diabetes, as well as the unfolded protein response markers in the cardiovascular tissues of the mice (Zhao et al, 2015). In a parallel study, conducted in the same laboratory with the same diet (5% SBP w/w), it has been shown that oral administration of SBP effectively reduced monocyte adhesion, as well as the anti-inflammatory stress in cardiovascular tissues of the mice (Zhao et al, 2014). These results confirmed the potential therapeutic effects of Saskatoon berries against diabetes metabolic complications, which are in many cases, the cause of death. However, such potentially beneficial effects have never been tested in humans.

2.4.6 Current state of knowledge on the health benefits of phenolic compounds

Despite the great deal of evidence supporting the beneficial health effects of the phenolic content of fruits, knowledge gaps exist with respect to cause and effect relationships. Most of the evidence on the benefits of phenolic compounds comes from *in vitro* and *in vivo* studies, and to a lesser extent from epidemiological research findings (Llorach et al, 2013; Velzen et al, 2014).

In an effort to explore the metabolic pathways and the corresponding physiological effects of polyphenols, bioavailability studies were conducted. They examined the metabolic changes and fates of the phenols from fruits or vegetables. The interpretation of the findings was always challenging due to changing circulating levels, the high degree of polyphenol

biotransformation, and the high concentration of plasma and urinary conjugated polyphenols. Such findings reflect the intensive metabolic changes taking place in the gut and the liver (Velzen et al, 2014). As such, it is necessary to broaden the scope of search for metabolites.

Potential interactions taking place between nutrients, nutraceuticals and the metabolome, makes the analyses of their intestinal, hepatic and urinary metabolism complex. However, suites of tools have advanced to facilitate these investigations. Recent advancements in food technology allow for novel food ingredients to be positioned for testing. Additionally, well-designed human clinical trials and associated analytical techniques to assess their outcomes, has made the study of particular food components, directly and indirectly, both convenient and reliable.

2.5 Dietary antioxidants, oxidative stress and Inflammation

The interest in the role of plant antioxidants as protective dietary components against inflammation is substantial. Oxidative stress and inflammation (both low-grade chronic and acute) are well linked with a wide range of the human complex diseases. The latter include cardiovascular diseases, diabetes and its complications, neurodegenerative diseases, cancer, intestinal inflammation and others. Since the occurrence of oxidative stress is possible without its development into inflammation, many experiments and epidemiological studies have provided evidence for its contribution to many of the chronic diseases. Chronic low-grade inflammation plays a crucial role in disease progression and pathogenicity (Arulselvan et al, 2016; Biswas, 2015; Satsangi et al, 2006).

Oxidative stress has been recently defined as “an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and/ or control

of molecular damage” (Biswas, 2015). Once oxidative stress has occurred, the consequences can vary depending on the extent of oxidation, antioxidant availability, tissues affected, and the overall health (Bjorklund and Chirumbolo, 2017; Biswas, 2015). Such consequences can include damage to cellular DNA, proteins, lipids or carbohydrates, eventually leading to impairment in function at the cellular level and subsequent cellular death and disease progression (Biswas, 2015; Devasagayam et al, 2004).

With respect to an established definition of inflammation, it is commonly considered a complex reaction in the vascularised connective tissue in response to exogenous and endogenous stimuli (Biswas, 2015). The goal of this response is to get rid of the causes and consequences of macromolecule and cellular injury events. Depending on the stimulus and the cellular mechanisms involved in the process, inflammation can be classified as either acute or chronic (Biswas, 2015).

Endogenous enzymatic and non-enzymatic antioxidant systems serve as the first line of defense against an imbalanced oxidative status. However, when the body is affected by stress, ROS production increases markedly. Under substantial oxidative stress, the endogenous protective mechanisms may be unable to restore this imbalance and prevent macromolecular damage (Arulselvan et al, 2016). Exogenous antioxidants are available from two sources: natural sources of phenolic compounds abundant in many fruits and vegetables, and synthetic sources. Examples of the latter include two commonly used antioxidants in the food industry: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Natural antioxidants are purported to have no significant side effects as compared to their synthetic counterparts. The current use of BHA and BHT, however is banned in most countries due to their association with cancer. Thus, natural antioxidants are considered the most valuable therapeutic agents against oxidative stress

and the associated inflammation. Evidence from both pre-clinical and clinical trials shows that phenolic compounds and flavonoids are especially effective as antioxidants and anti-inflammatory agents, exerting their properties by various mechanisms (Arulselvan et al, 2016; Biswas, 2015).

2.5.1 Inflammation and diseases

Inflammation is a series of events, taking place at the cellular level. Associated events include dilation of arterioles and venules, resulting in altered vessel permeability, and percolation of white blood cells into the affected tissue. An inflammatory event that is unable to reach resolution, overtime contributes to tissue damage, ultimately leading to death. Although inflammation is a natural response of the immune system, it can occur over prolonged periods of time. Chronic inflammation not yet reaching a state of resolution, along with other predisposing factors, can cause many of the chronic diseases such as inflammatory bowel disease, diabetes mellitus and others (Arulselvan et al, 2016).

2.5.2 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is a general term describing a group of inflammatory conditions of the small and the large intestines. The two major disease entities are Crohn's disease (CD) and ulcerative colitis (UC). Crohn's disease can be far more invasive and profound in nature as the chronic inflammation occurs in most of the parts of the gastrointestinal tract, including the oral cavity, stomach, intestines and the rectum. Whereas in the case of UC, it is normally the large intestine and/or the rectum that are the affected areas. As of 2015, IBD prevalence in the Western world (North America, Europe, Australia, and New Zealand) is 0.5%

of the population (Kaplan, 2015). In Manitoba, as of 2006, its prevalence is 15 cases per 100000, (Bernstein et al, 2006). The incidence of this disease, however, is increasing rapidly in the industrialized countries. Evidence from epidemiological studies suggests an increasing upward trend for the upcoming years (Kaplan, 2015). It is believed that the chronic inflammation affecting the gastrointestinal tract is the outcome of the interaction of genetic predisposition and environmental factors. Epidemiologic and genetic research has provided strong evidence that knowledge of genetic determinants may assist in preventative or therapeutic regimens capable of reducing disease severity. Different SNPs predispose to the disease, and of special interest are those of nutritional-relevance (Bernstein et al, 2006; Betteridge et al, 2013; Jarmakiewicz et al, 2017; Molodecky et al, 2011; Plevy et al, 2013).

The exact cause of IBD is not well defined at present. For this reason, the diagnosis is based on a combined approach. Results from clinical examination, endoscopy, histopathology and laboratory testing are used together to identify the presence of IBD. However, in some cases, the diagnosis results remain “uncertain”, requiring additional serological and genetic marker(s) for further confirmation (Plevy et al, 2013; Satsangi et al, 2006). The study of the molecular mechanisms to further understand the disease etiology is of great importance to deliver a precise diagnosis, treatment and a follow-up, and to improve disease outcomes and overall quality of life. To this point, it is important to test for potential genetic markers, and in order to do so, it is essential to identify them in the first place.

2.5.3 Genetics & IBD

Complex disease genetics is becoming an increasingly hot topic in biomedical research, thanks to genetic association study findings. The genetics implicated in CD and UC are no

exception. In 2011, 99 IBD susceptibility loci were identified, with 28 commonly associated with both disease entities: CD and UC, 71 loci associated with CD and 47 with UC (Lees et al, 2011). A closer look at the loci associated with IBD, identified only a few of them as nutritional relevance. Recently, an intronic SNP of the sodium-dependent ascorbic acid transporter gene *SLC23A1* was found to strongly associate with Crohn's disease (Shaghghi et al, 2014). It is worth noting that knowledge about relevance of loci to IBD susceptibility is rapidly growing. However, their actual mechanism(s) of action have yet to be revealed.

Based on the above mentioned association, it is highly probable that novel genetic markers for intestinal inflammation will be found among the ascorbic acid transporter family SLC23 genes. Two such candidate markers are *SLC23A2* and *SLC23A3*. Both genes are nominated based on the shared sequence homology with each other and with their family member *SLC23A1*. Sequence homology in this context refers to the nucleotide sequence of the gene transcripts (the coding sequence of the specified genes: *SLC23A2* and *SLC23A3*). To this point, a shared sequence is a cursor for similarity in gene products, in this case, the proteins encoded by *SLC23A2* and *SLC23A3*.

2.5.4 Single nucleotide polymorphism of the SCL23 and diseases

Nucleotide substitutions known as single nucleotide polymorphisms (SNPs) contribute to disease pathogenesis (Orr and Chanock, 2008). SNPs are nucleotide substitutions at a fixed position in the genome, resulting in different DNA sequences among subjects of the same ethnic group. Common genetic variations of the *SLC23* genes (SNPs of *SLC23A1* and *SLC23A2*) have been associated with disease predisposition. SNPs were shown to associate with gastric and intestinal diseases with an inflammation etiology. A group of case-control studies conducted on

several Caucasian cohorts showing the highest prevalence and incidence rates among all other population groups have been published (Betteridge et al, 2013). Within these studies (summarized in Table 2.1), associations were found between SNPs in *SLC23A2* and colorectal adenoma in a Caucasian cohort in the United States (Erichsen et al, 2008). Additionally, associations with gastric cancer in an EPIC European cohort (Duell et al, 2013), as well as a significant association found between one SNP in *SLC23A2* and the risk for gastric cancer in Caucasians of Poland (Wright et al, 2009) were identified. Recently, a SNP in *SLC23A1* associated with Crohn's disease vulnerability in a Canadian Caucasian cohort was reported (Shaghaghi et al, 2014).

Table 2.1: Summary of SNPs in *SLC23* genes associated with inflammatory gastrointestinal diseases

<i>Disease</i>	<i>Transporter gene</i>	<i>Allele (haplotype) or genotype</i>	<i>Population/ sample size</i>
<i>Crohn's disease</i> ¹	<i>SLC23A1</i> rs10063949	G/A: G carrier	Caucasians (Canada) 453
<i>Colorectal adenoma</i> ²	<i>SLC23A2</i> rs1110277 rs4987219	Diplotype G-C decreased risk (OR= 0.49) (strong linkage disequilibrium, D' 0.89; 95% CI= 0.86-0.92)	Caucasians (United States) 4834
<i>Gastric cancer</i> ³	<i>SLC23A2</i> rs6116569	C/T	Caucasians (Europe) 1649
<i>Gastric cancer</i> ⁴	<i>SLC23A2</i> rs12479919	G/A	Caucasians (Poland) 693

¹ Shaghaghi et al (2014)

² Wright et al (2009)

³ Erichsen et al (2008)

⁴ Duell et al (2013)

The associations between genetic variations and diseases can always be examined for a given gene, even though the gene is not characterized. *SLC23A3* is the third member and orphan gene of the SLC23 gene family that is not well characterized. A review of the available literature on SLC23A3 highlighted five published peer-reviewed articles in the PubMed central library.

Two of these were comprehensive review papers with details of the SLC23 family, including the classification, function, tissues of expression and substrates. The reports focused on SLC23A1 and SLC23A2 genes and proteins, and referred to the orphan member, namely SLC23A3 (Brüzle and Hediger, 2012; Brüzle et al, 2013). The other two reported genetic association study findings. Schizophrenia has recently been shown to associate significantly with the 2q35 locus in the Japanese population. In this study, the human *SLC23A3* locus was considered a candidate marker for disease susceptibility, as pinpointed in a few studies conducted over the past ten years (Rantapriya et al, 2010; Shibata et al, 2013). Shibata, et al (2013) found that the most significant schizophrenia-associated SNP was located in the upstream region of *SLC23A3* to be rs13404754. Contrary to the significant association found between the *SLC23A3* SNP and schizophrenia, the functional candidate gene failed to correlate with juvenile epilepsy, as shown in the other report (Rantapriya et al, 2010). One report recently showed that SLC23A3 expressed in Caco-2 cells correlated with an increased apical membrane area (Nikulin et al, 2018).

SLC23A1 and SLC23A2 are well-characterized genes and proteins (Daruwala et al, 1999; Erichsen et al, 2001; Eck et al, 2003). Despite the significant knowledge available on the genomics and genetics of SLC23A1 and SLC23A2, less is known, about the impact of the genetic variation of *SLC23A2* and *SLC23A1* on health in general. The reason for this lack of knowledge relates to the fact that the identified variations were found to be non-functional. Hence, they are at least not under consideration for further research. Genetic variations are associated with different diseases, and investigating these variations is a promising strategy to better help prevent and cure several complex diseases.

Genome databases predict that *SLC23A3* transcripts have 12 splicing patterns, 5 of which are believed to encode proteins. A putative splice variant of 610 amino acids long is predicted to

be the main isoform, translated from the 2010 base pairs long transcript (namely: SLC23A3-203), with 12 coding exons (ENSEMBL, 2014). *SLC23A3* is not characterized on a genomic or functional level, as no publication record exists.

2.5.5 Dietary management of IBD

The successful management of IBD during all stages requires individualized diet plans that are regularly evaluated and reinforced. While diet is the main behavior manipulated by people with IBD, in most of the published guidelines for IBD treatment and management, the role of diet therapy is minor, or even missing. Despite the difference in disease patterns, genetics and histology among CD versus UC patients, all IBD patients are normally given similar diet plans and dietary recommendations. With the exception of the known effectiveness of enteral therapy (Halmos and Gibson, 2015; Olendzki et al, 2014), diet therapy regimens given to IBD patients have included increased fluid intake, reduced fat and fiber intake, and reduced intake of dairy products (for lactose intolerant patients) (Mishkin, 1997; Olendzki et al, 2014). A great deal of evidence calls for a more effective involvement of nutrition and diet, as an integral part of the treatment plan for patients with IBD (Halmos and Gibson, 2015; Olendzki et al, 2014). Since oxidative stress and inflammation are clearly involved in the etiology and progression of IBD, it is prudent to include antioxidant-rich foods as a treatment option. Recently, there has been many reports on the efficacy of non-traditional therapy for IBD, such as the administration of dietary antioxidants from different sources including vitamin A, vitamin C and phenolic compounds (Moura et al, 2015).

2.6 Diabetes mellitus

According to the American Diabetes Association, diabetes is defined as “a group of metabolic diseases characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels” (American Diabetes Association, 2010).

In Canada, in 2009 it has been estimated that one in ten adults will face premature death as a result of diabetes complications. A worldwide prevalence of 6% for diabetes mellitus type 2 was estimated in 2007 (246 million people), and it is expected to rise to 7.3% by 2025 (Perera and Li, 2012). According to the Canadian Diabetes Association’s estimates of 2015, 22.1% (5.7 million) of the Canadian population aged 20 years and over and 23.2% (6.4 million) are predicted to be pre-diabetics in 2015 and 2025, respectively. It is predicted that diabetes prevalence will increase from 9.3% (3.4 million) in 2015 to 12.1% (5 million) in 2025. Estimates are available on the diabetes charter for Canada’s website

(<https://www.diabetes.ca/getmedia/513a0f6c-b1c9-4e56-a77c-6a492bf7350f/diabetes-charter-background-national-english.pdf.aspx>).

Diabetes mellitus can be easily managed by the successful integration of drugs, lifestyle changes and, most importantly, diet. There is a general agreement that a low glycemic index diet can help control blood glucose levels, as well as blood lipid profiles. This suggests its role in the treatment of diseases related to insulin resistance. More specifically, epidemiological evidence suggests also that low glycemic index diets may reduce type 2 diabetes mellitus risk (Ley et al, 2014). The most likely underlying mechanism behind the latter observation may be the lower insulin demand of such diets. There is a consensus, approved by the World Health Organization

and the Food and Agriculture Organization, that recommends consuming more of the low glycemic index foods (Alfens et al, 2005; Lobos et al, 2017; Perera and Li, 2012).

2.6.1 Dietary management of diabetes

Medical nutrition therapy is an integral part of the self-management and treatment of diabetes mellitus. Nutrition therapy aims at improving quality of life and nutritional and physiological health, and mitigate the associated consequences and complications. This can be achieved through glycemic control. However, there is no one-size-fits all eating pattern. It can be challenging to choose the appropriate foods and determine how much and how often to eat for persons with diabetes (American Diabetes Association, 2018; Sievenpiper et al, 2018).

It is well agreed that monitoring carbohydrate intake, keeping in mind the corresponding postprandial blood glucose levels, are vital for glucose control and associated clinical outcomes. Persons with type 2 diabetes of all ages are strongly recommended to focus on consuming complex carbohydrates from whole grains, fruits, vegetables and dairy products (especially milk and yogurt). Moreover, they are also advised to reduce their intake of simple and refined carbohydrates (American Diabetes association, 2018; Sievenpiper et al, 2018). To this point, the use of functional foods of a low glycemic index, containing low amounts of simple carbohydrates, as well as diets rich in antioxidants would be promising.

2.7 Functional foods

‘Functional foods’ as a term first appeared in 1993, in an article entitled “Japan explores the boundary between food and medicine”. It is a market-related term, where the information generated by research findings comes into practice (Doyon and Labrecque, 2008). Functional

foods have many definitions, although none of them is recognized by law worldwide. According to Health Canada (<http://www.agr.gc.ca/eng/industry-markets-and-trade/canadian-agri-food-sector-intelligence/functional-foods-and-natural-health-products/?id=1170856376710>), a functional food is “similar in appearance to conventional food, consumed as part of the usual diet, with demonstrated physiological benefits, and/or to reduce the risk of chronic disease beyond basic nutritional functions”. According to the International Life Sciences Institute of North America, functional foods are “foods that by virtue of physiologically active food components provide health benefits beyond basic nutrition” (Henry, 2010). Based on the available literature, one can think of a functional food as being a food item to which a component has been added.

2.7.1 Current state of knowledge on the health benefits of functional foods

The mechanisms by which various metabolic pathways are altered by diet or particular foods within the diet are rapidly becoming more apparent. This is particularly due to advancements in analytical approaches that possess increased sensitivity and accuracy. Extensive knowledge is being generated about nutrients, and the routes of entry and exit of metabolites from the body. Over the past few years, both nuclear magnetic resonance and mass spectrometry-based metabolomics techniques have been used in nutritional interventional studies, for both chronic and acute phase exposures. From an analytical perspective, plasma/serum and urine samples are the choice in most of the cases with few studies analyzing fecal samples. Plasma analysis provides a broad picture of the metabolic changes that occur in the circulation at a certain time point and typically reflects the response of the metabolome to a diet or food. Urine is analyzed as a non-invasive biological fluid for the measurement of certain biomarkers, metabolites or specific nutrients (Hanhineva et al, 2015; Rio et al, 2013).

Very few trials have been conducted to assess the metabolic responses towards a functional food made up of a dairy product fortified with a plant extract. While many dietary interventional trials have been conducted utilizing metabolite profiling analyses that explore differential dietary intake over extended time durations, few trials exist on acute dietary exposures (post-prandial).

In 2015 Hanhineva and colleagues published a comprehensive review of the dietary interventional studies conducted between 2009 and 2015 that utilized metabolomics approaches. The authors reviewed a total of 44 original research papers, each research study aimed at assessing the effects of dietary interventions of whole diets, particular foods or individual compounds on the human metabolome. In general, either healthy volunteers or individuals with specific diseases (example: metabolic syndrome) were sampled for urine, plasma, feces or a combination, and the samples were analyzed using nuclear magnetic resonance, or mass spectrometry coupled with gas or liquid chromatographic separation. Of particular interest, six studies were evaluated. Each study was designed to test foods or nutrients that pertain to the target functional food in this proposed research, that is Saskatoon berry yogurt (SBY). (1) A clinical trial looked at the metabolic responses of ingesting acidified milk products in a group of irritable bowel syndrome patients and their matching controls (Pedersen et al, 2010). Patients consumed 400 ml of milk (probiotic fermented milk versus non-probiotic acidified milk) every day, over a period of 8 weeks. Proton nuclear magnetic resonance (^1H NMR) metabolomics analysis was performed. Results showed identical effects of acidified milk independent of probiotic addition. The findings were supported by a questionnaire-based evaluation of symptom relief (Pedersen et al, 2010). However, this study lacked the existence of a control for the acidified milk, which may have been the reason for a lack of difference between the treated

groups. (2) Another study utilized resonance mass spectrometry metabolomics analysis and genotyping as an integrated approach to examine the link between genetic and metabolic profiles of vitamin D and calcium supplementation for 3 months (Elnenaei et al, 2011). Blood and urine samples were analyzed from 56 postmenopausal women. Participants were classified into 3 groups based on their bone density assessed by heel ultrasound. Study participants included low density responders, low density non-responders and controls. An important finding was that folate metabolism has been suggested to play a role in calcium and vitamin D status, and the body's response to supplementation (Elnenaei et al, 2011). (3) An NMR-based metabolomics approach was used to examine the differential metabolic profiling of the urine and feces of healthy subjects who consumed milk or cheese, and their effect on blood cholesterol levels (Zheng et al, 2015). 15 participants were involved in a crossover clinical trial, participants consumed either a control isocaloric diet, a high milk isocaloric diet, or a high cheese isocaloric diet with equal amounts of calcium for 2 weeks. Findings showed that cheese consumption has resulted in an increased level of microbiota-related metabolites, compared to milk. Overall, dairy consumption showed a strong effect on the urine and fecal metabolome (Zheng et al, 2015). It was suggested that the increased production of short chain fatty acids might have contributed to the altered microbiota. (4) In another controlled dietary interventional trial, the effect of dairy products was determined. 67 overweight and obese participants consumed dairy products on daily basis, over a period of 12 weeks (Piccolo et al, 2015). One of the main objectives of the study was to identify responsiveness to energy restriction on weight loss (Piccolo et al, 2015). Findings showed a difference in fat loss (adiposity), and plasma stearate levels, irrespective of weight loss responsiveness. (5) A non-targeted metabolite profiling analysis was used in a study conducted by Hanhineva and co-workers (2015). The aim of the study was to examine the effect

of dietary modifications of an increased consumption of whole grains, fatty fish, and bilberries on the plasma metabolite profiles. 106 participants with metabolic syndrome participated in the study for over 12 weeks. Compared to the control diet, marked differences were observed in the fasting plasma metabolic profiles of the test food subjects. Novel biomarkers of selected foods were identified utilizing liquid chromatography mass spectrometry (Hanhineva et al., 2015). (6) In another randomized, crossover and controlled clinical trial, 20 volunteers consumed either 40 g/day of cocoa powder in 500 ml skimmed milk, or skimmed milk alone for 4 weeks. Urine samples were analyzed for full metabolic profiles utilizing high performance liquid chromatography-hybrid quadrupole time of flight negative and positive ionization modes (Llorach et al, 2013). The analysis revealed that 39 compounds were linked with cocoa intake but not with skimmed milk alone. The difference between the cocoa milk and the control interventions was marked (Llorach et al, 2013). The study concluded that the metabolic profile might be used to identify endogenous markers that link cocoa intake to cardiovascular disease risk.

Breakfast is an important meal. Most of the commercially available breakfast cereals, especially the ones not made with whole grain or high fiber ingredients, are examples of foods that are known for their ability to raise postprandial glucose and insulin levels rapidly. However, adding functional food ingredients or applying processing steps (such as fermentations) may lead to the production of low glycemic index foods. Glycemic index is defined as a property of carbohydrate-containing foods that provide a basis for predicting their postprandial blood glucose response. The glycemic response is controlled by many factors of the food intake, including the nutrient composition, the energy content, meal duration, palatability, volume and weight of the meal and the associated rheological attributes (Alfens et al, 2005). In one study, the

addition of fermented milk (yogurt) and pickled cucumber to a breakfast of a high glycemic index was shown to lower both the glycemic and insulinemic responses (Östman et al, 2001). The test food was given to a group of healthy subjects and was compared to regular milk and cucumber (Östman et al, 2001).

2.7.2 Saskatoon berry yogurt as a potential functional food

Functional foods hold the potential to enhance glycemic control in pre-diabetics or diabetics. Saskatoon berry is a promising ingredient, especially if it is added to low GI foods (such as yogurt). Dairy products are basic food items (fermented dairy products) on the Canadian table, especially at breakfast or as snacks. In one trial that examined the glycemic effect of a cereal-based breakfast, it was found that the inclusion of milk did not alter the glycemic index of the cereal. Knowledge pertaining to the glycemic indices of some fermented milk products is lacking. The same is also true for the insulinemic indices of milk products (Östman et al, 2001). Therefore, the purpose of the second study was to characterize a modified fermented dairy product rich with antioxidants and to specify the ingredients that attribute to its improvement of the glycemic index.

Recent advancements in analytical chemistry techniques have the potential to identify novel biomarkers in conjunction with functional food consumption. Studies are warranted to permit a comprehensive and detailed assessment of the chemical composition, and effects of Saskatoon berries on various metabolic responses in a sample of healthy participants in preparation for its use as a functional food.

2.7.3 Knowledge gap

Genetic predisposition to IBD is evident. However, few novel genetic biomarkers have been identified. An SNP identified in *SLC23A1* has been associated with IBD, and by virtue of tissue expression and sequence homology, variants in *SLC23A2* and *SLC23A3* are thus candidates for association with IBD. However, the association of *SLC23A2* and *SLC23A3* SNP variants with IBD has never been investigated. Hence, the potential association between SNP variants in *SLC23A2* and *SLC23A3* and IBD was addressed in the first research study.

The health benefits of Saskatoon berry, attributed to its phenolic content, are well documented in *in vitro* studies. Moreover, the glycemic effects of these berries are well documented in *in vivo* animal models. However, SBP has never been fully characterized. In addition, its nutritional value and phenolic content, both after being processed to powder and added to yogurt, are unknown. Furthermore, while human glycemic responses have been reported to be positively modulated after ingesting berries that have comparable phenolic profiles to Saskatoon berries, the latter have yet to be studied. Specifically, the glycemic and metabolic responses to the ingestion of SBY, a functional food, remain to be assessed. The glycemic and metabolic responses to the ingestion of SBY in a group of healthy participants was assessed in the second research study.

2.8 References

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Chapter 3: Objectives and Hypotheses

The current research included 2 distinct studies. The first study is entitled “Nutrigenetics of ascorbic acid and IBD”, and the second study is entitled “Metabolic responses to Saskatoon berry yogurt”. The specific objectives and hypotheses for these studies are presented below.

3.1 Objectives and hypotheses

The overall goal of this research was to understand the link between dietary antioxidants and health outcomes utilizing 2 distinct studies employing novel approaches. The first study is entitled “Nutrigenetics of ascorbic acid and IBD”. The second study is entitled “Metabolic responses to Saskatoon berry yogurt”. Each of the study outcomes, along with parallel research findings, will have potential preventive and/or therapeutic applications, including the elucidation of how to improve the antioxidant content of foods and how to maximize their utilization once in the body.

3.1.1 Objectives for Study #1

For the first study, namely “Nutrigenetics of ascorbic acid and IBD”, in order to test the proposed hypotheses, the following aims were determined:

- A) Genomic characterization of the orphan *SLC23A3* gene: Chromosomal locus, molecular size and sequence, exon intron structure, alternative splicing patterns, promoter region(s) and transcription factor binding sites.
- B) Genomic characterization of the putative SLC23A3 ascorbic acid transporter: Splice variants and membrane domains.

- C) Genotype selected *SLC23A3* SNPs in the Manitoba IBD cohort examined for associations with IBD.
- D) Genotype selected *SLC23A2* SNPs in the Manitoba IBD cohort examined for associations with IBD.

3.1.2 Hypotheses for Study #1

- A) The human *SLC23A3* gene is a putative ascorbic acid transporter gene.
- B) SNPs in the *SLC23A3* gene associate with intestinal inflammation in Caucasians.
- C) SNPs in the *SLC23A2* gene associate with intestinal inflammation in Caucasians.

3.1.3 Objectives for Study #2

For the second study, namely “Metabolic responses to Saskatoon berry yogurt”, in order to test the proposed hypotheses, the following aims were determined:

- A) Characterize the chemical composition and the phenolic profiles of SBP and SBY.
- B) Determine the effect of adding Saskatoon berry powder to the yogurt on the antioxidant capacity of SBY.
- C) Measure the glycemic index of SBY.
- D) Examine post-prandial plasma glucose, insulin and total antioxidant capacity of SBY.
- E) Investigate the phenolic metabolic responses to SBY in plasma and urine using QTOF mass spectrometry.

3.1.4 Hypotheses for Study #2

- A) The addition of Saskatoon berry powder contributes to the antioxidant capacity of yogurt.

- B) SBY is a low glycemic index food.
- C) SBY has an antioxidant capacity in the plasma of healthy participants.
- D) The consumption of SBY shows lower plasma glucose and insulin concentrations when compared to plain yogurt.
- E) The consumption of SBY contributes to the appearance of phenolic compounds in plasma and urine when compared to plain yogurt.

Chapter 4: *SLC23A3*: Genetic characterization, tissue expression, and susceptibility to intestinal inflammation

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Running title: *SLC23A3* characterization

Key words: *SLC23A3*, genomic structure, tissue expression, polymorphism, IBD

4.1 Abstract

The homeostatic balance of ascorbic acid is maintained in part by the solute carrier family 23 members: SLC23A1 and SLC23A2. Both are characterized Na⁺ dependent ascorbic acid transporters. However, the third member “SLC23A3” has yet to be characterized. The aim was to characterize “orphan” *SLC23A3* for the first time by describing the gene structure and examining tissue expression profiles. Additionally, another aim was to examine the link between *SLC23A3* variations and intestinal inflammation. Data sourced from gene databases were analyzed utilizing a bioinformatics approach. Gene expression was performed using RT qPCR. DNA samples of 288 CD cases, 265 UC cases and 269 matching controls were genotyped using RT PCR followed by binary logistic regression analysis. *SLC23A3* (8.63 kb in size) was located on 2q35 and exhibits three major splice variants encoding three protein isoforms (predicted to be 618, 610 and 493 amino acid residues in length) that vary in their transmembrane domains. Various tissues examined expressed *SLC23A3* mRNA. Among the epithelial tissues, renal, hepatic, and intestinal epithelial tissues showed significant levels of *SLC23A3* mRNA expression. Although *SLC23A3* mRNA is highly expressed in the intestinal tissues, none of the 4 selected SNPs in the *SLC23A3* were associated with IBD, among which was a rare variant rs79578215 on exon 2. *SLC23A3* variations of choice may not best represent the potential association to IBD.

4.2 Introduction

Solute carriers are gate-keepers for a wide range of nutrients and metabolites, localized on cellular membranes and in intracellular organelle membranes. One crucial role of solute carriers is the homeostatic regulation of their substrate solute(s). Two major sites of this regulation are intestinal absorption and renal reabsorption (Schlessinger et al, 2010). Solute carrier family 23 (SLC23) is a subset family of the solute carrier superfamily. Existing in all vertebrates, SLC23 is composed of three sodium dependent ascorbic acid transporters. SLC23A1 and SLC23A2 are well characterized and are responsible for the active transport of ascorbic acid across cellular membranes. The other member SLC23A3 is an “Orphan” member or the so-called nucleobase-ascorbate transporter 3 (Rajan et al, 1999; Stratakis et al, 2000; Wang et al, 2000; Burzle et al, 2013).

SLC23A1 and SLC23A2 were first cloned from a kidney cDNA library and functionality expressed by Daruwala and co-workers (1999). There is no published information available on the cloning of SLC23A3. The genetic characterization, functional properties, and physiology of SLC23A1 and SLC23A2 including tissue expression and distribution of the transporters and the role of these transporters in ascorbic acid regulation was reviewed by Burzle et al (2012; 2013). In addition, these authors linked the significance of a well-maintained ascorbic acid level and human health with genetic variations of SLC23A1 and SLC23A2.

Shibata et al (2013) examined the association of *SLC23A3* variants with schizophrenia susceptibility loci across the human genome utilizing thousands of microsatellite markers. It was found that rs13404754, located in the upstream region of *SLC23A3*, was significantly associated with schizophrenia. In contrast, whole genome linkage analysis in an Asian family of three generations showed that *SLC23A3* was not a potential candidate gene variant for juvenile

epilepsy (Ratnapriya et al, 2010). In another report, aimed to investigate arbutin genotoxicity on the differential gene expression in the human melanoma cell line (A375), DNA microarray analysis showed that *SLC23A3* was significantly up-regulated (Cheng et al, 2007). Although, Zhao et al (2010) reported that *SLC23A3* was a candidate locus associated with normalized pediatric height in European Americans, it was shown to play no role in pediatric stature determination in the population investigated. Most recently, the level of *SLC23A3* expression was correlated with an increased area of the apical membrane in Caco-2 cells (Nikulin et al, 2018). Despite the above studies, *SLC23A3* has not been fully characterized on a genomic or a functional level.

Tissue expression patterns and levels of expressed sequence tags (EST) can predict that a gene product is expressed and functioning in a given tissue. The intestinal epithelial expression of SLC23A1 and its role as an ascorbic acid influx transporter is well documented (Maulén et al, 2003; Wilson et al, 2005). In addition, *in vitro* studies (Eck et al., 2013) have demonstrated that renal epithelia express SLC23A1 and mediate ascorbic acid influx into proximal tubule cells. However, the ascorbic acid efflux transporter remains undefined. SLC23A3 shares tissue expression patterns with SLC23A1 and gene sequence homology as family members. Additionally, variant rs10063949 of the *SLC23A1* gene was associated with Crohn's disease in Caucasians (Shaghaghi et al, 2014). Based on the tissue expression profiles shared between SLC23A1 and its family member SLC23A3, it is hypothesized that *SLC23A3* variants may associate with intestinal inflammation.

In this study, the human solute carrier 23 member 3 gene (SLC23A3) was identified and characterized, in terms of its genomic locus and neighborhood, gene structure, major splice variants, protein isoforms and tissue expression profiles. In addition, the potential association

between single nucleotide polymorphisms (SNPs) spanning the *SLC23A3* gene and intestinal inflammation was examined, in a group of Caucasian inflammatory bowel disease (IBD) patients.

4.3 Methods

4.3.1 *SLC23A3* in silico analyses and genomic structure

Data on the human *SLC23A3* was sourced from two genome databases between May and June 2015. Data extracted from the gene database provided by the National Center for Biotechnology Information (NCBI) included the following information: Chromosomal locus and gene neighborhood, genomic region, reference sequence, nucleotide sequence of mRNA transcripts, transcription products (differential splicing patterns), polymorphisms, expression sequence tags (EST) tissue expression data, experimental gene expression data, and gene expression omnibus (GEO) data. Data extracted from the gene database provided by the European Bioinformatics Institute and the Wellcome Trust Sanger Institute (ENSEMBL) included the following information: Genomic region overview, region in detail, gene description (solute carrier family 23, member 3), transcript comparison, and splice variants comparison.

In silico analysis was performed utilizing the Sequencher® version 5.1 (a nucleic acid sequence analysis software tool). Analysis was performed for human *SLC23A3* {Genome Reference Consortium Human build 38 (GRCh38)} in terms of: Genomic structure, nucleotide sequence of transcripts, promoter region and transcription factors binding sites, polymorphisms, amino acid sequence of splice variants, and protein transmembrane domains.

4.3.2 Transmembrane domain prediction analysis for major protein isoforms

TMHMM server version 2.0 protein structure prediction tool (<http://www.cbs.dtu.dk/services/TMHMM/>) was used for the analysis of the transmembrane domains predicted for the *SLC23A3* gene products representing the three major protein isoforms (NM_001144890.1, NM_001144889.1 and NM_144712.4 ordered according to amino acid length respectively).

4.3.3 *SLC23A3* tissue expression polymerase chain reaction

Tissue expression of the human *SLC23A3* was done with real time qPCR, performed on a StepOnePlus thermal cycler (Applied Biosystems), operated by the StepOne software tool version 2.2.2. The software was used for data analysis. PCR was performed utilizing TaqMan based qPCR assay (Applied Biosystems) to determine the levels of the *SLC23A3* transcripts in human tissues. Human intestinal, intestinal segments, hepatic, and renal marathon ready cDNA from Clontech Laboratories (Table 4.1) were used in the qPCR. qPCR was performed using assays in the TaqMan GTXpress™ master mix (4401892) (Applied Biosystems) according to manufacturer's specifications. Assays were performed in triplicate for each of the cDNA samples. The following thermal cycle conditions were used: 95°C for 20 seconds for initial denaturation followed 40 cycles of 95°C for 3 seconds and for 50°C for 30 second. Data were processed using Step One v2.2.2 software tool. A Standard curve was generated from the plasmid clone IDMHS1010-7507872 (Applied Biosystems) representing the major *SLC23A3* transcript, which was used in serial dilutions to produce the standard curve. Plasmid copy numbers were calculated using the equation: $\text{Number of copies} = (\text{amount} * 6.022 \times 10^{23}) / (\text{length} * 1 \times 10^9 * 650)$ (<https://www.google.ca/#q=plasmid+copy+number>), with the help of the Excel

software tool.

Table 4.1: Human *SLC23A3* cDNA tissue identification information

	<i>cDNA tissue</i>	<i>Clontech® reference number</i>	<i>Lot number</i>
1	<i>Duodenum</i>	S1684	3030137
2	<i>Jejunum</i>	S1686	3030140
3	<i>Illeum</i>	S1688	3030139
4	<i>Illeocecum</i>	S1685	3030138
5	<i>Cecum</i>	S1689	3030142
6	<i>Colon Ascending</i>	S1681	3030134
7	<i>Colon Transverse</i>	S1683	3030136
8	<i>Colon Descending</i>	S1682	3030135
9	<i>Rectum</i>	S1687	3030141
10	<i>Liver</i>	S1691	3030144

4.3.4 *SLC23A3* SNPs and inflammatory bowel disease:

4.3.4.1 Participants

A total of 553 samples from IBD cases (288 Crohn's disease and 265 ulcerative colitis) from the Manitoba IBD cohort and 269 samples from matching controls were included in this study. Participants' ages were between 18 and 80 years old with a female: male ratio of 3:2. The vast majority of the participants were Caucasians (95%) and the average duration of disease was 5.4 years. The Manitoba IBD cohort study was initiated in 2002, recruiting persons who were 18 years or older, had been diagnosed with IBD for at least 7 years, and registered in the University of Manitoba IBD Research Registry (which was established in 1995) (Appendix A). The registry included residents of Manitoba (Canada) that are diagnosed with IBD as per the administrative database of Manitoba Health. More than half of the IBD patients in Manitoba took part in the registry. The Manitoba IBD cohort study was approved by the University of Manitoba Health

Research Ethics Board (REB), and all participants had provided a written consent form for involvement in the study. Additional details on the IBD cohort have been described earlier (Ediger et al, 2007; Rawsthorne et al, 2012).

4.3.4.2 DNA samples

The DNA samples were generously provided by the collaborators (IBD samples) Dr. Charles Bernstein and (matching healthy controls, without evidence of inflammation) Dr. Hani El-Gabalawy, both of the Rady Faculty of Health Sciences, University of Manitoba. The total number of case samples was 553, including 288 samples from participants diagnosed with Crohn's disease and 265 samples from participants diagnosed with ulcerative colitis. There were 269 matched control samples (matching by age and sex). DNA was extracted from the peripheral white blood cells using standard protocols mentioned previously (Packer et al, 2004). Briefly, venous blood samples were collected from IBD patients and controls, and samples were kept in EDTA tubes at 4°C until further analysis. For analysis, 5 ml of each blood sample was homogenized inside PCR amplification tubes, amplification reagents were added and the PCR was initiated. Once white blood cells appeared, an aliquot of 10 µl each sample was further homogenized, the supernatant was discarded, and the leukocyte pellet was washed with PCR buffer and stored at -20°C (Angelini et al, 2002).

4.3.4.3 SNP selection

SNPs in the sodium-dependent ascorbic acid transporter gene *SLC23A3* were determined by examination of the identified SNPs in the population of interest (Caucasians) which is

available on public databases including dbSNP (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) and NIH (<https://www.ncbi.nlm.nih.gov/snp>).

SNPs were selected across the gene at regular intervals in order to enhance the coverage of the full length of the gene. A total of four SNPs were chosen across the *SLC23A3* (genomic size: 8.27 kilo base pairs). 3 Tag-SNPs¹ were selected based on haplotype sequence analysis for the common variations (SNPs) and 1 rare SNP was selected for the orphan *SLC23A3*, based on the high frequency of occurrence in the population of interest. Rare SNPs are believed to be of higher biological importance (impact) than the common SNPs.

4.3.4.4 Genotyping

DNA samples were genotyped for the selected Tag-SNPs (selection criteria below, table 4.2). Genotyping was performed using TaqMan assays from Applied Biosciences® (real time polymerase chain reaction). The reaction conditions were set according to the optimized assays according to the manufacturer's protocol as per the conditions described below.

Thermal cycling conditions: Initial denaturation at 95°C for 30 seconds followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, extension at 68°C for 2 minutes, and final extension at 68°C for 5 minutes.

¹ Tag SNPs: SNP analysis of the SLC23 genes have shown haplotypes to be partitioned into blocks of high linkage disequilibrium. Genetic variation across the length of a block with several SNPs is represented as a whole using characteristic “Tag” SNPs to be genotyped in a given population of samples. This reduces the genotyping effort and cost in studies aimed at associating genetic variation to disease phenotypes

Table 4.2: SNPs details and TaqMan assay codes for the *SLC23A3* gene

<i>SNP ID</i>	<i>Position</i>	<i>Allele</i>	<i>Applied Biosciences® assay ID (TaqMan assays)</i>
<i>rs13404754</i>	5' UTR 219170859	A>G	C__1827490_10
<i>rs79578215 (rare)</i>	Exon 2 219169529	C>G	C__99967993_10
<i>rs6753739</i>	Intron 9 219164178	A>G	C__30257477_10
<i>rs6713887</i>	3' UTR 219161647	T>C	Made to order

4.3.4.5 Statistical design and analysis

To verify the associations between polymorphisms in the *SLC23A3* gene and IBD risk (Crohn's disease/ ulcerative colitis), SPSS statistical analysis software tool (version 23 for Mac) was used. Binary logistic regression analysis outputs were used to estimate the odds ratio (OR) for IBD phenotypes (Crohn's disease and ulcerative colitis), 95% confidence intervals (CIs) were used for the association between SNP genotypes and IBD risk (both phenotypes), adjusted for sex and age. As a reference group for the allelic occurrence, the common homozygous allele (for each of the SNPs) is considered the reference group.

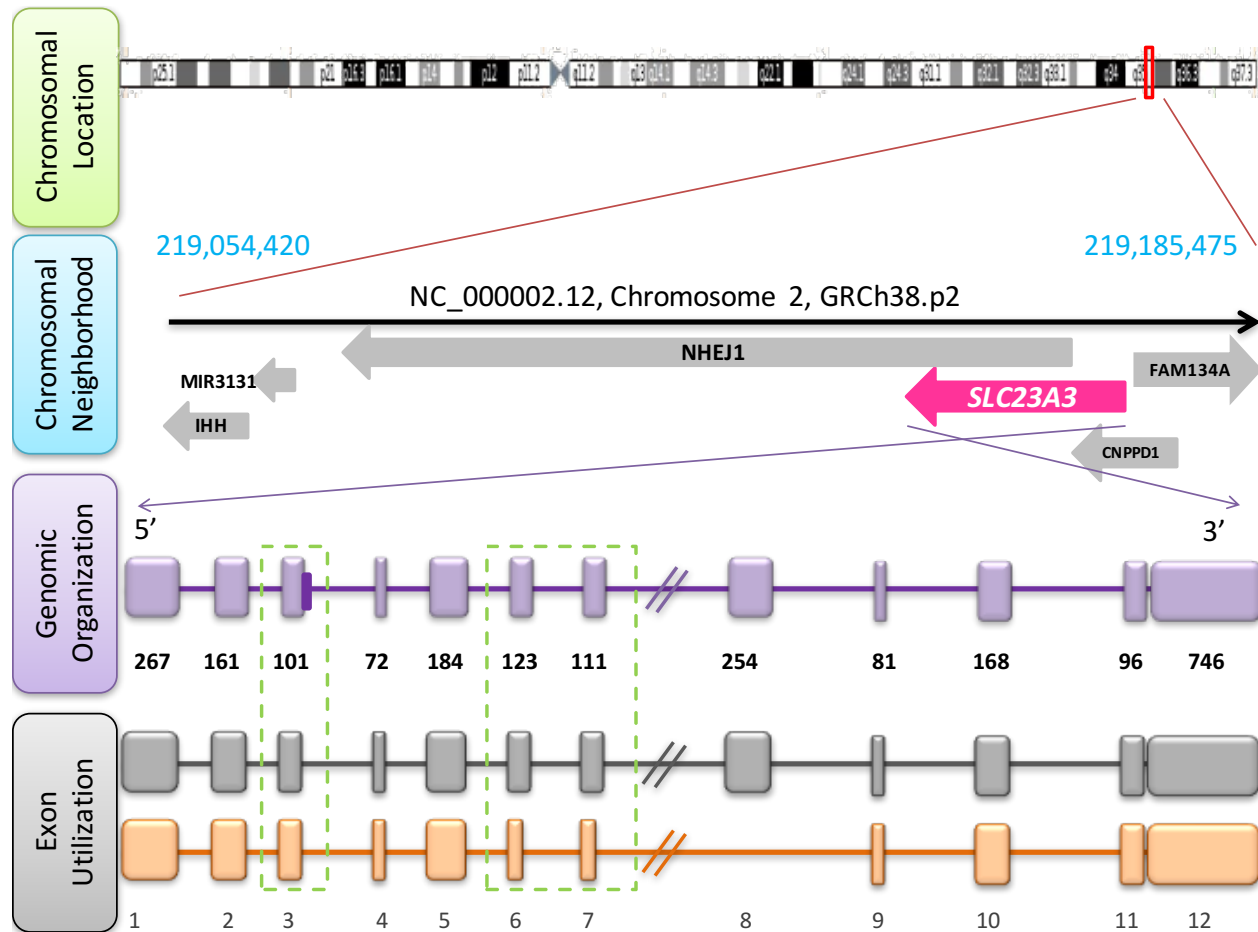
4.4 Results

4.4.1 Genomic locus and gene neighborhood

On the q arm of chromosome number two, the second in length among the human genome, sits the *SLC23A3* gene (Figure 4.1). Within approximately 130 kb flanking the gene, a non-homologous end joining factor 1 (NHEJ1) overlaps *SLC23A3* downstream and two other

regulatory elements exist downstream, namely microRNA 3131 (MIR3131) and Indian hedgehog signaling molecule (IHH), respectively. Upstream of the *SLC23A3* region exists the cyclin Pas1/ PHO80 domain-containing protein 1 (CNPPD1) and the family with sequence similarity 134 member A (FAM134A) on the sense genomic/ chromosomal strand. *SLC23A3* is found to span 8630 base pairs (bp) along the genomic sequence of chromosome 2 in between nucleotides 219161558 and 219170188.

Figure 4.1: Genomic structure overview of *SLC23A3*. Genomic location and splice variants: *SLC23A3* has a genomic size of 8.630 kilo bp. Flanking the gene in both directions are 5 regulatory elements/ coding proteins. *SLC23A3* is organized into 12 exons which are all coding regions and 11 introns which are all non-coding regions, 3 alternative splicing patterns exist



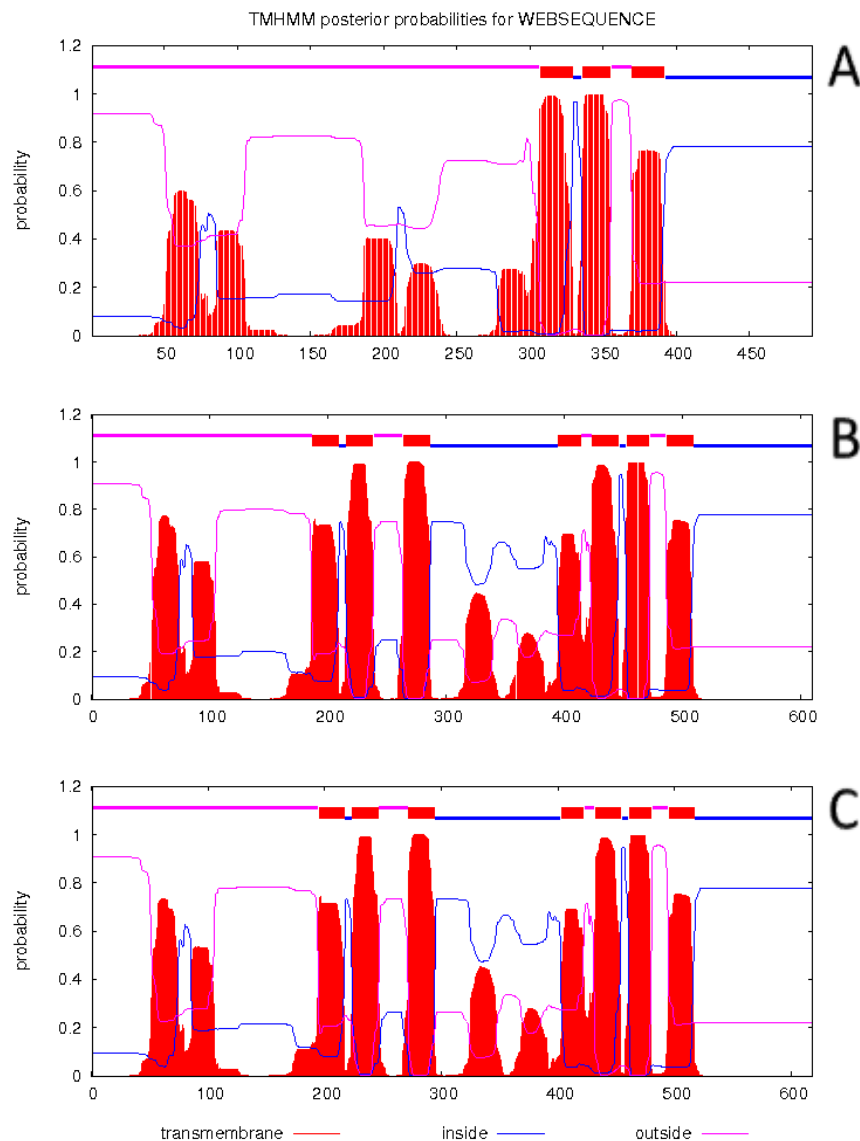
4.4.2 Gene structure and major variants

The gene is organized into 12 exons that are all coding regions and 11 introns that are all non-coding regions (Figure 4.1). The sequence of each exon was confirmed by manual curation of the aligned nucleotide sequence of each of the major transcripts (NM_144712.4, NM_001144889.1 and NM_001144890.1) with the genomic sequence available for *SLC23A3* (NC_000002.12). Apparently, three alternative splicing patterns exist for *SLC23A3*, giving rise to three structurally distinct transcripts. In the main full length transcript (figure 4.1, indicated in purple) there is an additional retained intronic piece adjacent to exon 3. This transcript is referred to as variant 3. The smallest transcript (figure 4.1, indicated in orange) is missing an exon (exon 8) and has a smaller size for exons 6 and 7 comparing to the first two transcripts.

4.4.3 *SLC23A3* protein structure prediction

Three distinct isoforms are predicted to be encoded by the three transcript variants differing in full sequence and ORF lengths. According to NCBI, transcript variant 1 (NM_144712.4, full mRNA sequence length: 2037 bp) ORF is 1482 bp long, and is predicted to encode for a 493 amino acid isoform as shown in figure 4.2A. Variants 2 and 3 are much similar (NM_001144889.1, full mRNA sequence length: 2010 bp and NM_001144890.1, full mRNA sequence length: 2048 bp). ORFs are 1833 and 1857 bp long, and are predicted to encode protein isoforms of 610 and 618 amino acids in length, respectively (Figure 4.2B and C). Predicted membrane topology for both of the isoforms (variants 2 and 3) is much similar, and is predicted to have 11 transmembrane domains. For transcript variant 1, a total of 8 domains are predicted for the small 493 amino acids long isoform.

Figure 4.2: Membrane protein topology depiction of SLC23A3 isoforms. A: Reference isoform {NM_144712.4} transmembrane domains shown in red bars/ peaks of transcript variant 1, predicted to be 493 amino acids in length, representing 8 transmembrane domains. B: Reference isoform {NM_001144889.1} transmembrane domains shown in red bars/ peaks of transcript variant 2, predicted to be 610 amino acids in length, representing 11 transmembrane domains. C: Reference isoform {NM_001144890.1} transmembrane domains shown in red bars/ peaks of transcript variant 3, predicted to be 618 amino acids in length, representing 11 transmembrane domains. The additional amino acids and transmembrane domains in the variants 2 and 3 are predicted to change the membrane topology, and to be functional proteins



4.4.4 Tissues expressing *SLC23A3*

A combined model approach was used to determine the tissue expression patterns for *SLC23A3*. Qualitative expression data available in gene database was used to reflect specific tissue distribution and approximate expression profiles for *SLC23A3*, utilizing two pieces of evidence: The AceView data and the EST/ UniGene expression data (as reported by sequence submitters). Additionally, real time qPCR was utilized to further confirm and quantify the expression patterns in two of the epithelial tissues of special importance to the solute of interest “potentially ascorbic acid” homeostasis. These included the intestinal segments and the hepatic tissues.

From RNA sequencing data, two sets of data values are depicted: The Aceview data and the EST/ UniGene data. In the Aceview data set, the values expressing RNA presence were normalized as significant Fragments Per Kilobase of exon per Million fragments mapped (sFPKM). The level of *SLC23A3* RNA expression is shown in Figure 4.3 (A). The highest expression was observed in testis and lowest in brain. The EST data in Figure 4.3 (B) presents the *SLC23A3* RNA expressed sequence tags (EST) in a given tissue per million transcripts examined. High expression patterns can be observed in all tissues with the kidney showing the highest expression profile.

The RT qPCR experimental findings are shown in Figure 4.3 (C). Human intestinal segments (duodenum, jejunum, ileum, ileocaecum, caecum, ascending colon, transverse colon, descending colon and the rectum) and the liver were examined for *SLC23A3* expression profiles based on the tissue expression pattern shared between the *SLC23A3* and its family member *SLC23A1*. The highest expression levels represented by expression profiles were seen in the liver, ileum, ileocaecum and the ascending colon, respectively.

Figure 4.3 (A): Bars showing *SLC23A3* expressed in tissues in significant fragments per kilobase of exon per million fragments mapped (sFPKM). Various body tissues are shown, the colon and the kidney demonstrate high expression, and are considered major sites of solute cellular uptake and reabsorption, respectively

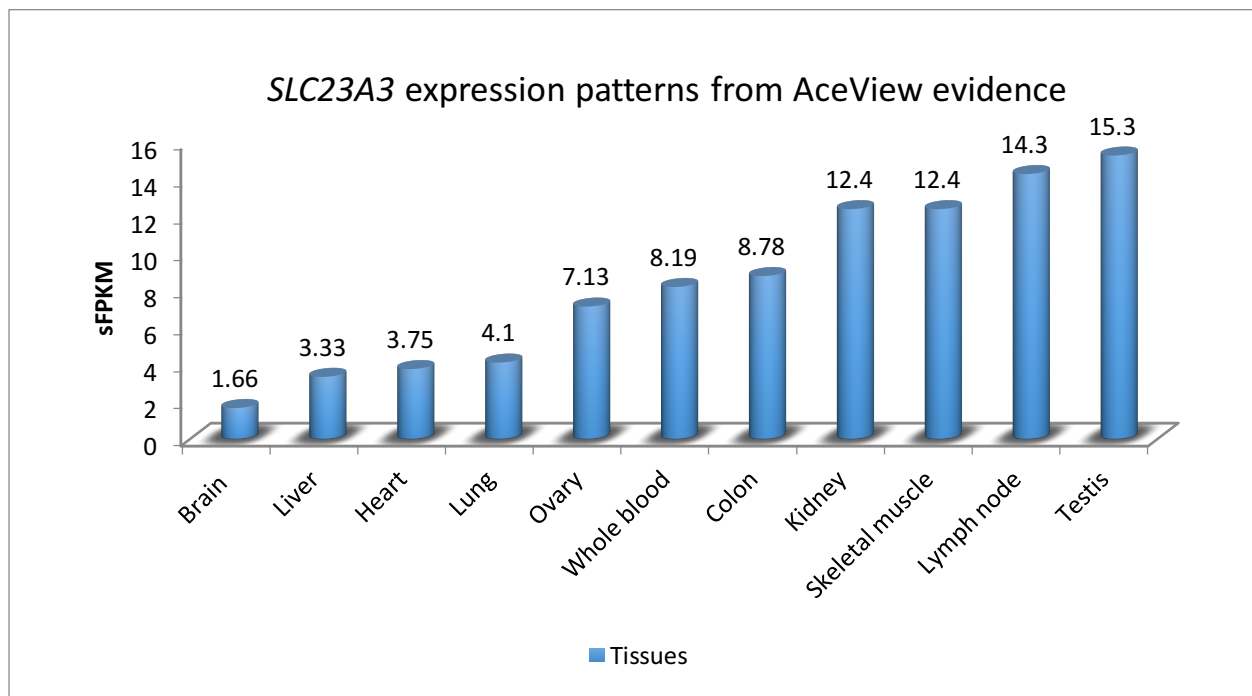


Figure 4.3 (B): Bars showing *SLC23A3* expressed in tissues in significant transcripts per million. Various body tissues are shown, the kidney tissue demonstrates then highest expression, and is considered the major site of solute cellular reabsorption

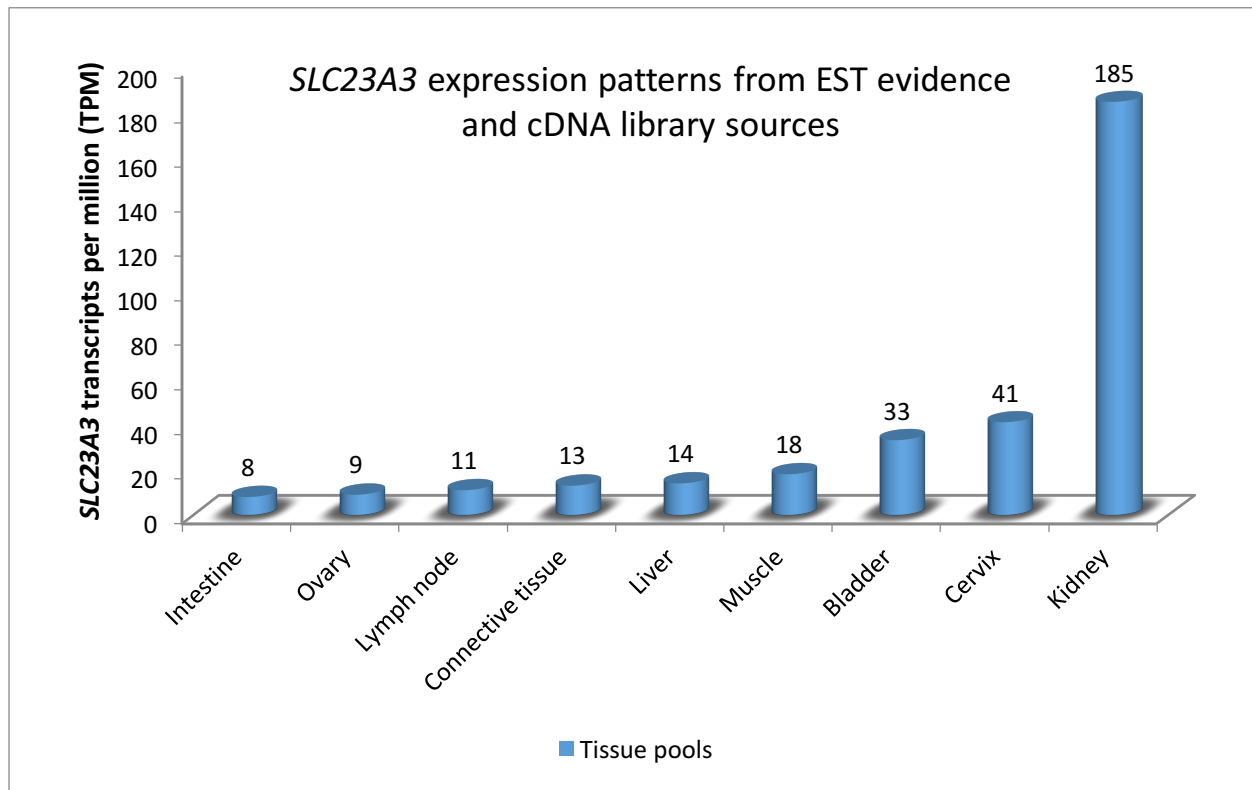
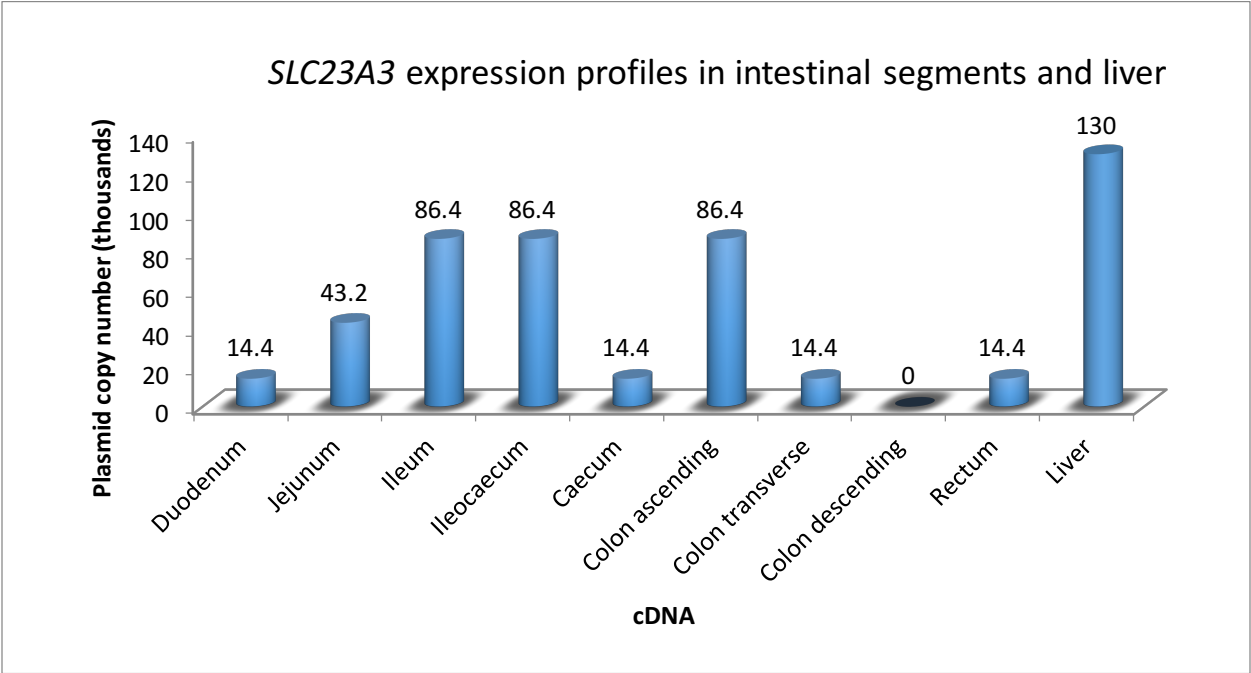


Figure 4.3 (C): Bars showing quantitative *SLC23A3* expression profiles in intestinal and hepatic tissues



4.4.5 SLC23A3 variants and susceptibility to IBD

Genomic DNA samples from a total of 822 participants of the Manitoba IBD cohort study were included. The ages of participants of the IBD study were between 18 and 80 years old, and 53.7% were female IBD cases and 46.3% were male IBD cases. The IBD study participants were previously described in full detail (Ediger et al, 2007; Rawsthorne et al, 2012). In this study, we have extended the sample using the same study cohort to overcome the problem of a small sample size and to enhance the power analysis. A subset sample (less than 450 participants) was recently described in some details in terms of the disease location and behaviour (Shaghghi et al, 2014). Table 4.3 shows the baseline characteristics of the IBD cohort.

SNP genotype frequency data for CD and UC are shown in table 4.4 and table 4.5, respectively. Four SNPs of the *SLC23A3* were genotyped, 3 of which are common SNPs (rs13404754, rs6753739 and rs6713887) and 1 rare SNP (rs79578215) with an allele frequency of 0.01 in healthy Caucasians. None of the selected SNPs were associated with either CD or UC.

Table 4.3: Baseline characteristics of study participants *

<i>Characteristic</i>	<i>Crohn's disease (n = 288)</i>	<i>Ulcerative colitis (n = 265)</i>	<i>Controls (n = 269)</i>
	<i>n (%)</i>	<i>n (%)</i>	<i>n (%)</i>
<i>Sex</i>			
<i>Female</i>	156 (54.2)	141 (53.2)	170 (63.2)
<i>Male</i>	132 (45.8)	124 (46.8)	99 (36.8)

*No significant difference in baseline characteristics observed between participants.

Table 4.4: *SLC23A3* genotype and allele frequencies in CD and controls

<i>SNP/ allele</i>	<i>Crohn's disease (n = 288)</i>	<i>Controls (n = 269)</i>	<i>Odds ratio (95% CI)</i>	<i>P value</i>
	<i>n (%)</i>	<i>n (%)</i>	<i>n (%)</i>	
<i>rs13404754</i>				
<i>AA</i>	132 (45.83)	134 (49.81)	Reference	
<i>AG</i>	47 (16.31)	44 (16.36)	1.03	0.89
<i>GG</i>	113 (39.24)	111 (41.26)	1.02	0.94
<i>rs79578215</i>				
<i>CC</i>	288 (100)	269 (100)	Reference	
<i>CG</i>	0 (0)	0 (0)	ND*	ND
<i>GG</i>	0 (0)	0 (0)	ND	ND
<i>rs6753739</i>				
<i>AA</i>	134 (46.53)	141 (52.42)	Reference	
<i>AG</i>	121 (42.01)	108 (40.15)	0.86	0.40
<i>GG</i>	33 (11.46)	20 (7.43)	0.61	0.11
<i>rs6713887</i>				
<i>TT</i>	211 (73.26)	130 (48.33)	Reference	
<i>TC</i>	121 (40.01)	100 (37.17)	0.81	0.24
<i>CC</i>	38 (13.19)	39 (14.50)	1.01	0.97

*ND: not determined.

Table 4.5: *SLC23A3* genotype and allele frequencies in UC and controls

<i>SNP/allele</i>	<i>Ulcerative colitis (n = 265)</i>	<i>Controls (n = 269)</i>	<i>Odds ratio (95% CI)</i>	<i>P value</i>
	<i>n (%)</i>	<i>n (%)</i>	<i>n (%)</i>	
<i>rs13404754</i>				
AA	78 (29.43)	91 (33.83)	Reference	
<i>AG</i>	133 (50.19)	134 (49.81)	0.89	0.54
<i>GG</i>	54 (20.38)	44 (16.36)	0.72	0.19
<i>rs79578215</i>				
CC	265 (100)	269 (100)	Reference	
<i>CG</i>	0 (0)	0 (0)	ND*	ND
<i>GG</i>	0 (0)	0 (0)	ND	ND
<i>rs6753739</i>				
AA	131 (49.43)	141 (52.42)	Reference	
<i>AG</i>	114 (43.02)	108 (40.15)	0.89	0.53
<i>GG</i>	20 (7.55)	20 (7.43)	0.99	0.98
<i>rs6713887</i>				
TT	106 (40)	130 (48.33)	Reference	
<i>TC</i>	116 (43.77)	100 (37.17)	0.70	0.06
<i>CC</i>	43 (16.23)	39 (14.50)	0.74	0.24

*ND: not determined.

4.5 Discussion

In this report, the solute carrier 23 member 3 gene (*SLC23A3*) is described for the first time. *SLC23A3* is mapped to band 35 on the long q arm of chromosome two. *SLC23A3* has a genomic size of 8.63 kb. It is the smallest family member in terms of genomic length, compared to 16.1 and 158.4 kb, corresponding to *SLC23A1* and *SLC23A2*, respectively (Eck et al, 2003). *SLC23A2* and *SLC23A1* are twenty and two times larger than *SLC23A3*, respectively, which indicates a substantial difference in the gene product size. However, *SLC23A2* and *SLC23A1* are only 5 and 3 exons larger, respectively, than the 12 exons of *SLC23A3*.

Three distinct protein isoforms are encoded by this single gene. Prediction analysis indicate 11 transmembrane domains for the larger variants 2 and 3, and only 8 domains for variant 1. Such variety indicates the possibility for differential expression and/or functionality. The high number of transmembrane domains indicates that these isoforms are putative membrane proteins. Functional characterization needs to be performed to confirm this prediction.

Several human tissues expressed *SLC23A3* mRNAs, and according to the findings of this study, these tissues encompass most tissue types. The brain expressed the least amount of *SLC23A3* while the testis expressed the highest amount as determined by the AceView data. However, according to data from the EST/ UniGene, the kidney had the highest level of expression. Gene database evidence (AceView and UniGene), along with the findings of this study demonstrate that *SLC23A3* is expressed in epithelial tissues of critical importance, including intestinal, hepatic (AceView, UniGene, data from this study) and renal tissues (AceView and UniGene data), and may contribute to overall ascorbic acid homeostasis (Corpe et al, 2010; Michels et al, 2013). Interestingly, this pattern of expression of *SLC23A3* in the absorptive and reabsorptive epithelia of the intestines and the kidney, respectively, is very similar to its family member *SLC23A1* which encodes the sodium dependent ascorbic acid

transporter SLC23A1. SLC23A1 mediates ascorbic acid influx into cells and contributes, in part, to overall increased plasma ascorbate levels (Corpe et al, 2010; Michels et al, 2013). *SLC23A1* is highly expressed in the small intestine, liver, kidney, lung, and the pancreas. Expression of *SLC23A1* in the proximal kidney tubule is the most important contributor to the regulation of vitamin C in the body. *SLC23A1* is involved in vitamin C renal reabsorption following glomerular filtration of plasma (Eck et al, 2003; Michels et al, 2013). SLC23A1 is the primary ascorbic acid active transporter and controls the bulk of absorption and reabsorption (Eck et al, 2003; Corpe et al, 2010). SLC23A1 is responsible for ascorbic acid influx into enterocytes and renal epithelial cells across the apical side (at the brush-border of intestinal and renal lumina) (Michels et al, 2013; Wilson et al, 2005).

The next step was to gain more knowledge about the specific *SLC23A3* expression profiles in absorptive epithelia. Specifically, expression profiles in the small and large intestinal segments were of particular interest. RT qPCR experiments performed in the laboratory showed that *SLC23A3* was more substantially expressed in the ileum, ileocaecum, and the ascending colon than in the other segments. Additionally, the highest expression was shown in hepatic epithelial tissues. According to the evidence from gene databases (AceView), *SLC23A3* is substantially expressed in the colon, which is parallel to the findings of this study. *SLC23A3* is also highly expressed in the kidney (AceView and UniGene). Drawing on the tissue expression patterns observed for *SLC23A1*, these findings suggest a “potential” role of *SLC23A3* in intestinal absorption and renal reabsorption.

A comparative look at *SLC23A3* and *SLC23A1* reveals that both genes are closer to each other in size than their family member *SLC23A2*. Additionally, comparative homology analysis of protein structure shows that SLC23A3 shares a 34% amino acid sequence with SLC23A1

(Burzle et al, 2012). These findings, along with the expression profiles among the SLC23 family members, may suggest that SLC23A3 is a putative ascorbic acid transporter. Further experiments are required to delineate SLC23A3 functionality.

Genetic variations in the *SLC23A3* gene have not been extensively investigated. In this study, four variants spanning the *SLC23A3* gene were looked into, two of which are located in the regulatory regions: The promoter region (5' UTR) and the 3'UTR, namely: rs13404754 and rs6713887, respectively. Although *SLC23A3* is highly expressed in the colon, none of the selected SNPs associate with IBD.

On a different note, one of the chosen SNPs (rs79578215) to be tested in relation to IBD sits on exon 2, a coding region of the gene, with a very low allele frequency overall. This rare variant did not exist in any of the cases or the controls of this study. Further exploration will reveal more information on the potential role of genetic variations of the *SLC23A3* gene during health and disease.

Recognizing the link between the genetic variation of *SLC23A3* and health outcomes might be of clinical relevance. Furthermore, the genetic characterization of *SLC23A3* lays the background for future findings. On a different note, the sample size used in this study, although enough to produce valid results, was not ideal. The findings cannot be generalized until further confirmed, regardless the sample size.

4.6 Conclusion

In conclusion, this is the first report to describe the genomic structure for *SLC23A3*. *SLC23A3* is the smallest gene among its family members, with a confirmed genomic size of 8.6 kilo bp. Three major SLC23A3 protein isoforms exist and vary in length and transmembrane

domain structures: 618, 610, and 493 amino acids each. *SLC23A3* is significantly expressed in intestinal, hepatic, and renal tissues. The latter shows the highest *SLC23A3* expression levels. *SLC23A3* is a putative ascorbic acid transporter. Unlike *SLC23A1*, SNPs in *SLC23A3* are not associated with intestinal inflammation in Caucasians.

4.7 References

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Transition statement 1

Data from Chapter 4 represent the first report to confirm the genomic structure of *SLC23A3* and to examine the disease association with IBD of *SLC23A3* variants. *SLC23A3* is the smallest gene among its family members, with 2 possible functional isoforms at the cellular and molecular levels. Findings in relation to tissue expression patterns/ levels suggest that the *SLC23A3* could be a putative ascorbic acid transporter gene. However, more studies are needed to test its subcellular localization, substrate specificity, tissue expression, and function.

One of the main objectives of this thesis was to further investigate the SLC23 family, especially in terms of the impact of dietary antioxidants on inflammation. Hence, one of the aims was to test for the genetic variations of the *SLC23A2* in relation to intestinal inflammation. It is well known that systemic levels of ascorbic acid are usually lower in patients with IBD. This occurs during all stages of the disease, including both active episodes and remission (Ioannidis et al, 2011). Solute carriers SLC23A1 and SLC23A2 are well known to play an important role in regulating plasma ascorbic acid levels, but most importantly is the role of SLC23A2, which is the most abundantly expressed and distributed ascorbic acid transporter in the body. Recently, an intronic SNP of the ascorbic acid transporter SCL23A1 gene was found to associate with Crohn's disease (Shaghghi et al, 2014).

SLC23A2 variations have been associated with several colorectal, gastric, and colonic inflammation-related disorders. However, this has never been tested in IBD. Therefore, in the following chapter, will take a closer look at the variants of the *SLC23A2* in relation to susceptibility to IBD in a group of Caucasians, which is by far the most affected ethnicity with the highest prevalence rate of IBD worldwide (Betteridge et al, 2013).

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**Chapter 5: 3' UTR variant rs35560557 in the ascorbic acid transporter gene *SLC23A2*
associates with ulcerative colitis and Crohn's disease**

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5.1 Abstract

CD and UC are the two most common types of intestinal inflammation both showing an upward prevalence worldwide. CD and UC are multifactorial conditions, characterized by non-specific inflammation of the intestines. Many genetic variations such as SNPs predispose individuals to CD and UC and the search for SNPs that are relevant to oxidative stress in CD and UC is ongoing. *SLC23A2* helps protect against oxidative stress as an ascorbic transporter and a few SNPs in *SLC23A2* have been linked to predisposition of a range of gastric and intestinal inflammatory disorders. Therefore, this study aimed to determine if there was an association between *SLC23A2* variants and intestinal inflammation (CD and UC). DNA samples of 288 CD cases, 265 UC cases and 269 matching controls were genotyped using real-time PCR followed by binary logistic regression analysis. 3'UTR rs33560557 variant (GA genotype) and (AA genotype) increased susceptibility to UC 3.4 and 3.49 folds, respectively ($P < 0.01$), and (GA genotype) increased susceptibility to CD 2.93 folds ($P < 0.01$). This polymorphism may play a functional role in maintaining altered tissue/ plasma levels of ascorbic acid in IBD patients which might lead to oxidative stress and inflammation.

5.2 Introduction

Ulcerative colitis (UC) and Crohn's disease are the two main disease entities of Inflammatory Bowel Disease (IBD). In developed countries, IBD affects 1 in 200 people (Cleynen et al, 2016). The incidence rate has been estimated to be 20 cases per 100,000 people globally (Molodecky et al, 2011). In the Canadian province of Manitoba, the incidence rate has been estimated at 15 cases per 100,000 people (Bernstein et al, 2006). Epidemiological evidence suggests an increasing upward trend for the near future, as the age of disease onset is shifting from late adulthood to adolescence and early adulthood, therefore, the increase in incidence rates is exponential (Jarmakiewicz et al, 2017; Kaplan, 2015; Molodecky et al, 2011). Both UC and CD are characterized by a non-specific inflammation of the intestines causing nutrient mal-absorption and immunity disturbances. IBD and its associated complications have a great impact on the patient's quality of life, especially during the active stage of the disease (Lönnfors et al, 2014).

IBD is a multifactorial disorder with a genetic predisposition. Dietary habits and other environmental factors play an integrated role in determining the etiology of this disease. It is believed that the main pathophysiology behind IBD is an impaired immune response to enteric bacteria, and this leads to high levels of oxidative stress with subsequent inflammation of the gut (Scott et al, 2013). The exact cause of IBD is not well defined and the diagnosis is based on a combined approach. Results from clinical examination, imaging, endoscopy, histopathology, and laboratory testing are used together to identify IBD (Scott et al, 2013). However, in some cases, the diagnostic results remain "uncertain" and additional serological marker(s) may be required for further confirmation (Plevy et al, 2013; Satsangi et al, 2006). Furthermore, genetic markers can provide adjunctive information and additional diagnostic information (Scott et al, 2013).

Evidence from epidemiological studies and genome-wide association studies positions several IBD-susceptibility single nucleotide polymorphisms (SNPs) and loci. Among the various endogenous susceptibility factors, SNPs may be the most relevant with respect to maintaining an overall oxidative balance. This may be especially true if the SNPs are present within the ascorbic acid transporter genes. Ascorbic acid is the main systemic non-enzymatic antioxidant that quenches various reactive oxygen species (May, 1999). It gives rise to the relatively stable semihydroascorbate radical which suppresses oxidative stress and the relevant inflammatory events (Pizzino et al, 2017). Immunomodulatory and corticosteroids drugs are not always successful treatment options. Since oxidative stress and inflammation are crucial in IBD initiation and progression, the use of dietary antioxidants such as vitamin C has been utilized recently to modify inflammation in IBD patients (Loannidis et al, 2011; Yan et al, 2015). The exact role of ascorbic acid in the management of IBD is not well understood. Yan et al. (2015) suggested that ascorbic acid reduces oxidative stress and inflammation in a colitis-induced mouse model. In their experiment, ascorbic acid increased the activity of enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase. Additionally, ascorbic acid inhibited the activation of tumor necrosis factor kappa B.

Plasma levels of ascorbate are reduced in IBD patients where mean levels of 51 μM are observed (Hengstermann et al, 2008). This is in contrast to healthy subjects where a circulating level of 30-60 μM of ascorbate is normally observed reaching a maximum of no more than 120 μM (Aguirre and May, 2008). Ascorbic acid homeostasis is maintained to a greater extent by SLC23A1 and SLC23A2. SLC23A1 serves as the main ascorbate transporter in the small intestine (Corpe et al, 2010). A genetic variant of SLC23A1 has been associated with Crohn's

disease vulnerability in a Canadian Caucasian cohort. However, specific mechanisms involved in this have yet to be revealed (Shaghghi et al, 2014).

SLC23A2 is ubiquitous in the body. It is responsible for the maintenance of intracellular ascorbic acid levels vital for cellular function and the protection against oxidative stress (Erichsen et al, 2008; Li and Schellhorn, 2007). SLC23A2 plays a crucial role in ascorbic acid accumulation in tissues as removal of the gene resulted in an elimination of ascorbic acid in most of the body tissues in an *slc23a2* knock-out mouse model (Erichsen et al, 2006). Genetic variations of the *SLC23A2* gene have been implicated in many gastric and intestinal complications and all are involved in inflammation. A group of case-control studies was conducted on several Caucasian cohorts. This is a demographic which shows the highest prevalence and incidence rates of IBD among all other population groups (Betteridge et al, 2013). Associations were found between SNPs in *SLC23A2* and several pathologies. These included colorectal adenoma in a Caucasian cohort of the United States (Erichsen et al, 2008), gastric cancer in the EPIC European cohort (Duell et al, 2013), and the risk for gastric cancer in Caucasians of Poland (Wright et al, 2009). Drawing on the present evidence, the expectation is that genetic variations of the *SLC23A2* gene may be linked with IBD. Genetic variation in the *SLC23A2* gene has never been examined for IBD susceptibility. This study aimed to test whether selected SNPs spanning the *SLC23A2* gene modulate susceptibility to IBD.

5.3 Methods

5.3.1 Participants

A total of 553 samples from IBD cases (288 Crohn's disease and 265 ulcerative colitis) from the Manitoba IBD cohort and 269 samples from matching controls were included in this study. Participants' ages were between 18 and 80 years old with a female: male ratio of 3:2. The vast majority of the participants were Caucasians (95%) and the average duration of disease was 5.4 years. The Manitoba IBD cohort study was initiated in 2002, recruiting persons who were 18 years or older, have been diagnosed with IBD for at least 7 years, and registered in the University of Manitoba IBD Research Registry (which was established in 1995) (Appendix A). The registry included residents of Manitoba (Canada) that are diagnosed with IBD as per the administrative database of Manitoba Health. More than half of the IBD patients in Manitoba took part in the registry. The Manitoba IBD cohort study was approved by the University of Manitoba Health Research Ethics Board (REB), all participants had provided a written consent form for involvement in the study. Additional details on the IBD cohort have been described earlier (Ediger et al, 2007; Rawsthorne et al, 2012).

5.3.2 DNA samples

The DNA samples were generously provided by the collaborators (IBD samples) Dr. Charles Bernstein and (matching healthy controls, without evidence of inflammation) Dr. Hani El-Gabalawy, both of the Rady Faculty of Health Sciences, University of Manitoba. The total number of case samples was 553, including 288 samples from persons diagnosed with Crohn's disease and 265 samples from persons diagnosed with ulcerative colitis. There were 269 matched control samples (matched by age and sex). DNA was extracted from the peripheral white blood

cells using standard protocols mentioned previously (Packer et al, 2004). Briefly, venous blood samples were collected from IBD patients and controls, samples were kept in EDTA tubes at 4°C until further analysis. 5 ml of each blood sample was homogenized inside PCR amplification tubes, amplification reagents were added and the PCR was initiated. Once white blood cells appeared, an aliquot of 10 µl each sample was further homogenized with reagents, the supernatant was discarded, and the leukocyte pellet was washed with PCR buffer and stored at -20°C (Angelini et al, 2002).

5.3.3 SNP selection

SNPs in the sodium-dependent ascorbic acid transporter gene *SLC23A2* were identified by looking through the known SNPs in the population of interest (Caucasians) available on the public databases including dbSNP (<https://www.ncbi.nlm.nih.gov/projects/SNP/>) and NIH (<https://www.ncbi.nlm.nih.gov/snp>).

SNPs were selected across the gene at regular intervals in order to enhance the coverage of the full length of the gene. A total of four SNPs were chosen across the *SLC23A2* (genomic size: more than 150 kilo base pairs). Tag-SNPs² were selected based on haplotype sequence analysis for the common variations (SNPs) of the *SLC23A2* and were based on the high frequency of occurrence in the population of interest.

² Tag SNPs: SNP analysis of the SLC23 genes have shown haplotypes to be partitioned into blocks of high linkage disequilibrium. Genetic variation across the length of a block with several SNPs is represented as a whole using characteristic “Tag” SNPs to be genotyped in a given population of samples. This reduces the genotyping effort and cost in studies aimed at associating genetic variation to disease phenotypes.

5.3.4 Genotyping

DNA samples were genotyped for the selected Tag-SNPs (selection criteria below, table 5.1). Genotyping was done using TaqMan assays from Applied Biosciences® (real time polymerase chain reaction). The reaction conditions were set according to the optimized assays according to the manufacturer's protocol as described below.

Thermal cycling conditions: initial denaturation at 95°C for 30 seconds followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, extension at 68°C for 2 minutes, and final extension at 68°C for 5 minutes. Table 5.1 below shows the selected SNPs matched with their TaqMan assays.

Table 5.1: SNPs details and TaqMan assay codes for the *SLC23A2* gene

<i>SNP ID</i>	<i>Position</i>	<i>Allele</i>	<i>Applied Biosciences® assay ID (TaqMan assays)</i>
<i>rs12479919</i>	Intron 1 4940094	C>T	A_006673
<i>rs1715365</i>	Intron 3 4918442	C>T	A_006671
<i>rs4987219</i>	Intron 8 4884300	C>G	001_0436
<i>rs35560557</i>	3' UTR/ exon 17 4854248	G>A	001_0454

5.3.5 Statistical design and analysis

To verify the association between polymorphisms in the *SLC23A2* gene and IBD risk (Crohn's disease/ ulcerative colitis), SPSS statistical analysis software tool (version 23 for Mac) was used. Binary logistic regression analysis outputs were used to estimate the odds ratio (OR)

for IBD phenotypes (CD and UC), 95% confidence intervals (CIs) were used for the association between SNP genotypes and IBD risk (both phenotypes), adjusted for sex and age. As a reference group for the allelic occurrence, the common homozygous allele (for each of the SNPs) is considered the reference group.

5.4 Results

Genomic DNA samples from a total of 822 participants of the Manitoba IBD cohort study were included. The ages of participants of the IBD study were between 18 and 80 years old, and 53.7% were female IBD cases and 46.3% were male IBD cases. The IBD study participants were previously described in full detail (Ediger et al, 2007; Rawsthorne et al, 2012). In this study, we have extended the sample using the same study cohort to overcome the problem of a small sample size and to enhance the power analysis. A subset sample (less than 450 participants) was recently described in some details in terms of the disease location and behaviour (Shaghaghi et al, 2014). Baseline characteristics of the IBD cohort are provided (table 5.2).

As the current study was designed as a pilot study, the main purpose of the research was to screen for associations and to identify potential biomarkers. Other than information on the participants' age, sex, phenotype, and SNP genotype, the participant's demographics are not meant to affect the findings nor influence the interpretation of the findings.

Genotype and allele frequencies of the selected *SLC23A2* SNPs in CD and UC patients are listed in table 5.3 and table 5.4, respectively. Data are expressed as odds ratio, with confidence intervals set at 95% confidence level and a *P* value of < 0.05 considered statistically significant.

This is the first study to investigate the association between selected variants of the sodium-dependent ascorbic acid transporter gene *SLC23A2* and the risk of intestinal inflammation in persons with both UC and CD. Among the 4 selected SNPs, genetic variant rs35560557 is located on the last exon of the *SLC23A2* gene. Compared to the rs35560557-GG homozygote carriers (reference allele), the rs35560557-GA heterozygous carriers were 2.9 times more at risk for CD ($P < 0.01$). Interestingly, the same variant poses 3.4 and 3.5-fold greater risk to UC ($P < 0.01$) among the rs35560557-GA heterozygous and the rs35560557-AA homozygous carriers, respectively. More than 21% of the CD cases carried the rs35560557-GA heterozygous allele, while 20% and 2% of the UC cases carried the rs35560557-GA heterozygous and the rs35560557-AA homozygous alleles, respectively. None of the other variants associated with IBD in this given cohort.

Table 5.2: Baseline characteristics of study participants*

<i>Characteristic</i>	<i>Crohn's disease (n = 288)</i>	<i>Ulcerative colitis (n = 265)</i>	<i>Controls (n = 269)</i>
	<i>n (%)</i>	<i>n (%)</i>	<i>n (%)</i>
<i>Sex</i>			
<i>Female</i>	156 (54.2)	141 (53.2)	170 (63.2)
<i>Male</i>	132 (45.8)	124 (46.8)	99 (36.8)

*No significant difference in baseline characteristics observed between participants.

Table 5.3: *SLC23A2* genotype and allele frequencies in CD and controls

<i>SNP/ allele</i>	<i>Crohn's disease (n = 288)</i>	<i>Controls (n = 269)</i>	<i>Odds ratio (95% CI)</i>	<i>P value</i>
	<i>n (%)</i>	<i>n (%)</i>	<i>n (%)</i>	
<i>rs12479919</i>				
CC	90 (31.25)	98 (36.43)	Reference	
<i>CT</i>	150 (52.08)	130 (48.32)	0.80 (0.55-1.15)	0.23
<i>TT</i>	48 (16.66)	41 (15.24)	0.78 (0.47-1.30)	0.35
<i>rs1715365</i>				
CC	69 (23.96)	75 (27.88)	Reference	
<i>CT</i>	166 (57.65)	143 (53.16)	0.79 (0.53-1.18)	0.25
<i>TT</i>	53 (18.40)	51 (19.00)	0.89 (0.53-1.47)	0.64
<i>rs4987219</i>				
CC	75 (20.04)	87 (32.34)	Reference	
<i>CG</i>	137 (47.57)	131 (48.70)	0.86 (0.58-1.28)	0.47
<i>GG</i>	76 (26.39)	55 (20.45)	0.65 (0.41-1.04)	0.08
<i>rs33560557</i>				
GG	211 (73.26)	138 (51.30)	Reference	
<i>GA</i>	61 (21.18)	117 (43.49)	2.93 (2.01-4.27)	< 0.01
<i>AA</i>	16 (5.56)	14 (5.20)	1.33 (0.63-2.83)	0.45

Table 5.4: *SLC23A2* genotype and allele frequencies in UC and controls

<i>SNP/allele</i>	<i>Ulcerative colitis (n = 265)</i> <i>n (%)</i>	<i>Controls (n = 269)</i> <i>n (%)</i>	<i>Odds ratio (95% CI)</i> <i>n (%)</i>	<i>P value</i>
<i>rs12479919</i>				
CC	92 (34.72)	98 (36.43)	Reference	
<i>CT</i>	130 (49.06)	130 (48.32)	0.93 (0.64-1.36)	0.71
<i>TT</i>	43 (16.23)	41 (15.24)	0.92 (0.55-1.53)	0.74
<i>rs1715365</i>				
CC	80 (30.19)	75 (27.88)	Reference	
<i>CT</i>	142 (53.58)	143 (53.16)	1.07 (0.73-1.59)	0.72
<i>TT</i>	43 (16.23)	51 (19.00)	1.27 (0.76-2.12)	0.37
<i>rs4987219</i>				
CC	65 (24.53)	87 (32.34)	Reference	
<i>CG</i>	138 (52.08)	131 (48.70)	0.74 (0.49-1.10)	0.13
<i>GG</i>	62 (23.40)	55 (20.45)	0.67 (0.41-1.10)	0.11
<i>rs33560557</i>				
GG	208 (78.49)	138 (51.30)	Reference	
<i>GA</i>	51 (19.52)	117 (43.49)	3.40 (2.30 -5.04)	< 0.01
<i>AA</i>	6 (2.26)	14 (5.20)	3.49 (1.31-9.31)	< 0.01

5.5 Discussion

The Manitoba IBD cohort comprises 95% of its participants as Caucasians living in Canada. In the current study, 4 SNPs were genotyped for intestinal inflammation association testing. 3 SNPs were located within the non-coding region (intron 1, 3, and 8) and 1 SNP located on the 3' UTR (exon 17). Subjects with the A-allele (both the rare homozygous genotype AA and the common heterozygous genotype GA) of the 3' UTR variant rs35560557 had a significantly higher risk for ulcerative colitis (almost 3.5-fold) than the G-allele carriers (reference allele). Likewise, carriers of the A-allele (heterozygous GA genotype) had 2.9 times more risk for Crohn's disease occurrence compared to the G-allele carriers (reference allele). Variant rs35560557 is located on the 3' untranslated region of exon 17 of the gene. SNPs located within the 3' UTR region in the human genome are rare (Ryan et al, 2014).

Although the exact cause of inflammation in UC and CD patients is still not fully elucidated, there is a consensus that a disturbed cellular antioxidant balance is a major contributor to the inflammatory events seen in IBD patients. Furthermore, ascorbic acid, as the main non-enzymatic antioxidant and is regulated, in part, through transport via SLC23A2 along with SLC23A1 (Lindblad et al, 2013). Both ascorbic acid transporters are expressed in the intestinal epithelia, where *SLC23A1* is targeted to the apical membrane and *SLC23A2* is targeted to the basolateral membrane of the cells (Hediger et al, 2012). According to the findings of this study, variant rs35560557 may contribute to the inflammatory events taking place in both the UC A-allele carriers (inflammation of the colon) and the CD A-allele heterozygous genotype GA carriers (non-localized inflammation of the intestines). Dietary ascorbic acid enters the enterocytes of the intestines through SLC23A1, and due to a possibly less functional SLC23A2, ascorbic acid accumulates inside the cells, reaching a concentration high enough to result in pro-

oxidant activity. Although the pro-oxidant action of ascorbic acid is generally mild, it may contribute to inflammation (Chakraborty et al, 2014).

SLC23A2 has a large genomic size of more than 150 kilo bp. A total of 2200+ SNPs have been identified and 32 different SNPs have been reported in study populations of various ethnic backgrounds. Most of the studied SNPs are located in the intronic (non-coding) regions of the gene and some are located in the untranslated regions of the gene (promoter 5'UTR and 3' UTR regions). Although the *SLC23A2* SNPs that have been analyzed do not fall within the exonic (coding) regions they may have a significant impact on *SLC23A2* expression levels in different cells (Michels et al, 2013).

Although the potential effects of SNPs located on the 3'UTR region of a given gene have not been extensively investigated to date, it is believed that SNPs located on the 3'UTR can have functional impacts. These may include microRNA binding and its subsequent effect on gene expression (Knox et al, 2018). A possible justification for the association between variant rs35560557 and IBD is the assumption that this SNP may result in an interruption of the post-transcriptional regulation of *SLC23A2* in the intestinal tissues. This includes the epithelial and the smooth muscle tissues of the colon (in case of UC) and both of the intestines (in case of CD) which would result in a reduced expression of *SLC23A2* or in the production of a transporter that is not fully functional. This would lead to an overall disturbed cellular antioxidant balance and thus inflammation. *SLC23A2* is expressed in almost every cell and tissue of the human body, with the exception of erythrocytes (Michels et al, 2013). This wide distribution of the gene is meant to protect metabolically active cells from oxidative stress and deliver ascorbic acid to tissues that have a demand for vitamin C as a cofactor (Bürzle et al, 2012). Despite this

knowledge on the tissue expression patterns of *SLC23A2*, very little is known about the tissue distribution of *SLC23A2* in humans (Duell et al, 2013).

In a study aimed at investigating the links between microRNAs of the human genome and some complex disease traits, a relatively rare SNP (variant rs16990301) of *SLC23A2* in a Yoruba African population appeared to influence ascorbic acid regulation. Levels of miR-30d microRNA correlated with decreased *SLC23A2* mRNA levels (Gamazon et al, 2012). The miRNAs are small (20-24 nucleotides) non-coding RNAs which play a role in gene silencing. They suppress gene expression by targeting complementary sequences on the 3' untranslated region of the mRNA to suppress the translation or to regulate the degradation or both (Knoz et al, 2018; Subramanian et al, 2018). Selected regions of the 3'UTR contain the miRNA binding site sequence. Therefore, a SNP located on the putative miRNA binding site(s) of the 3' UTR might have a functional role when examined using in silico analysis using available prediction models (Ryan et al, 2014; Knox et al, 2018).

Evidence from *in vitro* experiments shows that both the 3'UTR length, as well as the miRNA expression, are tissue specific (Ryan et al, 2014). These expression patterns are altered in many intestinal diseases including IBD and small bowel syndrome (Subramanian et al, 2018). UC and CD are both tissue-specific inflammatory disorders and there is a possibility for altered localized expression of *SLC23A2* miRNA and 3' UTR miRNA binding site(s) sequences. Both mechanisms could potentially cause an under expression of the *SLC23A2* leading to a reduced production of the transporter in tissues. The latter would progress to increased oxidative stress and the associated inflammatory events both systematically and in intestinal cells. Despite the limited knowledge on the tissue distribution of *SLC23A2*, many polymorphisms in the *SLC23A2* and *SLC23A1* are strongly associated with altered plasma levels of ascorbate, as well as tissue

concentrations of vitamin C (a role of *SLC23A1*). However, most of the screened SNPs are intronic or fall within the untranslated regions of the genes (Bürzle et al, 2012; Duell et al, 2013; Michels et al, 2013).

The main role that *SLC23A2* plays is in the management of oxidative stress and inflammation in metabolically active tissues (Duell et al, 2013). In IBD, the intestinal mucosal cells are in a chronic state of inflammation and this has been linked with a loss of 35 to 73% of ascorbate in the affected intestinal mucosa (Buffinton and Doe, 1995). Whether it is the reduced levels of ascorbate that cause mucosal inflammation or the mucosal inflammation that causes a reduction in ascorbate is not known. The understanding of the mechanisms by which variant rs35560557 and other variants cause physiological effects and subsequently contribute to the inflammatory events involved in the initiation and progression of IBD should be the goal for future research. These findings might be of relevance to the health care team when considering treatment options for people with both UC (mucosal inflammation) and CD (transmural inflammation) (Chambrun et al, 2010).

Although the sample size allows for accuracy in answering the research question, it is not the ideal sample size compared to other genome-wide association studies where a sample size of few thousands of each of the cases and their matching controls is analyzed. However, and regardless of the sample size, the findings cannot be generalized until further confirmed.

5.6 Conclusion

3'UTR variant rs35560557 (A-allele) associates with UC and CD, potentially altering cellular concentrations of ascorbic acid, antioxidant balance, and subsequently intestinal inflammation. The exact role of variant rs35560557 in the contribution to IBD has yet to be

determined. Due to its crucial role as a cellular antioxidant and free-radical scavenger, further studies into the regulation and expression of the ascorbic acid transporter gene *SLC23A2* in intestinal inflammation is of great importance and may hold promise in guiding therapeutic approaches.

5.7 References

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Transition statement 2

The overall goal of this research was to better explore the linkage between dietary antioxidants, inflammation, and health outcomes, utilizing models of the most recent advancements in research methodology. The main systematic dietary antioxidant, vitamin C, has been extensively investigated for its antioxidant role in healthy persons. And to a good extent, its antioxidant, as well as its anti-inflammatory properties were investigated in persons with intestinal inflammation and other non-localized inflammatory conditions. However, the role of ascorbic acid transporter gene variation has not been extensively studied, especially the ubiquitous transporter gene *SLC23A2*, in relation to intestinal inflammation. Taken together this knowledge gap, and the best use of the recent advances in research conduct, nutrigenomics best serves as the model of choice.

Ascorbic acid is an essential dietary antioxidant, with a well-established dietary recommendation for the healthy. Polyphenols are potent dietary antioxidants, that are not essential nutrients. Both ascorbic acid and polyphenols are well proven for their antioxidant and anti-inflammatory properties *in vitro* and *in vivo*. Due to the essentiality of ascorbic acid, its roles are addressed in human studies. This is not the case for the non-essential polyphenols, despite their potency. Polyphenols are less studied in healthy persons overall, and in persons with diseases in particular. To investigate the health benefits of such antioxidants like polyphenols, there is a need to start with healthy participants. The main goal of this research is to study the link between antioxidants and health outcomes. Testing the antioxidant potential of the non-essential polyphenols in healthy persons, requires that a high throughput technology be used. Metabolomics analyses is meant to detect primary and secondary metabolites, and serves best as a method of choice, when the objective is to search for metabolites as a result of food ingestion

(an antioxidant-packed food) or “potentially” a functional food.

In the previous chapter, the link between an essential dietary antioxidant (ascorbic acid) transporter variations and non-localized inflammation was investigated, using a nutrigenomics approach. A secondary model to explore this relationship includes the evaluation of the role of non-essential dietary antioxidants, specifically the polyphenols from Saskatoon berry, in relation to their anti-inflammatory properties.

In the first model “Nutrigenetics of ascorbic acid and IBD”, it was concluded that a 3’ UTR SNP (rs35560557) in the sodium-dependent ascorbic acid transporter gene *SLC23A2* significantly increased the risk of both UC and CD in Caucasians. Such a potentially functional SNP might affect the post-transcription of the gene as well as the functionality and the tissue distribution of the resulting protein in IBD. A deficiency of this study was the lack of data on vitamin C status. *SLC23A2* cellular sub-localization and tissue distribution experiments, along with genetic association studies looking into larger sample sizes, various ethnic backgrounds, and an assessment of vitamin C intake and status are required.

SLC23A2 regulates, in part, circulating levels of ascorbic acid. A primary or secondary deficiency of the vitamin can lower immune responses (Carr and Maggini, 2017). This can increase the susceptibility to inflammation-related diseases (Arulselvan et al, 2016). *SLC23A2* is expressed in all tissues, with the exception of erythrocytes. As *SLC23A2* contributes strongly to circulating plasma levels of this potent antioxidant, reductions in transport activity may predispose IBD patients to disease development, despite receiving adequate amounts of the vitamin from dietary and supplemental sources (Hengstermann et al, 2008; Michela et al, 2013). All IBD patients suffer a chronic, low-grade inflammation due to oxidative stress. In addition, such a state of oxidative stress and inflammation can contribute to other manifestations, such as

glucose intolerance, diabetes mellitus, metabolic syndrome, and various cancers, depending on other predisposing factors (Arulselvan et al, 2016; Haffner, 2006; Grivennikov et al, 2010).

Current research focused on understanding how dietary interventions, such as improving the nutritional status and how the provision of specific dietary bioactives/nutrients may influence immunity, oxidative stress, and inflammation, may potentially lead to a reduced risk of inflammatory diseases (Hanhineva, 2015; Ommen et al, 2017). Recent research has focused on the roles of phytochemicals found in plants and their extracts including those of fruits. Much is known about the antioxidant and anti-inflammatory properties of polyphenols *in vitro*. However, due to the lack of well-designed research models, little is known about the potency of dietary polyphenols as antioxidants and anti-inflammatory agents in humans (Velzen et al, 2014).

The knowledge about the mechanisms of how the various metabolic pathways are altered by diet or particular foods is accumulating rapidly, especially with the increased sensitivity and accuracy of the analytical procedures. This has led to the extensive knowledge about nutrients and their metabolites reaching the body through the diet and exiting the body (Rio et al, 2013; Velzen et al, 2014). Over the past few years, metabolomics-based techniques have been used in nutritional interventional studies of both chronic and acute phase exposures. Plasma and urine analytical samples are the choice in most of the cases with a few studies analyzing fecal samples. Plasma analysis provides a wide picture of metabolic changes occurring in the circulation at a certain time point and reflects the response of the metabolome to a diet or food. Additionally, urine is looked at when certain biomarkers, metabolites or specific nutrients are assessed (Hanhineva et al, 2015).

A secondary model was utilized, namely “Metabolic responses to Saskatoon berry yogurt”. In the following chapter, a targeted metabolomics approach was utilized, to analyze and

further investigate the effect of a group of dietary antioxidant polyphenols on the metabolic responses, in a group of healthy participants. The metabolic responses of glucose, insulin, and antioxidant capacity to some phenolic compounds and their derivatives/metabolites were examined. In addition, some primary and secondary metabolites of energy metabolism in plasma or urine were examined. The overall aim was to develop a functional food, Saskatoon berry yogurt (SBY) enriched with polyphenols, and to examine its potential as an anti-inflammatory functional food in a group of healthy participants. Yogurt, the food vehicle of choice in this study, is not only a low glycemic index food, but also, is proved to best retain phenolic compounds during frozen storage. Therefore, it was expected to best deliver Saskatoon berry phenolic compounds.

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Chapter 6: Effect of Saskatoon berry yogurt on postprandial glycemic, insulinemic and antioxidant responses in healthy participants

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6.1 Abstract

Berry phenolic compounds reduce glycemic effects and oxidative stress associated with diabetes in cell culture and animal models. The addition of Saskatoon berry powder (SBP) to a low glycemic index (GI) food such as yogurt may provide additional benefits to people with pre-diabetes. This study aimed to determine the effect of SBY on postprandial plasma glucose and insulin responses. Additionally, the antioxidant capacity of a given dose of SBP (19 g) and its associated postprandial antioxidant responses were examined. A randomized, controlled, cross-over trial was conducted with 12 healthy volunteers aged 18-35 years. Participants were given one of 3 foods containing 25 g of available carbohydrate at 4 visits in random order: 1) SBY; 2) Plain yogurt; 3) white bread (2 visits). Plasma concentrations of glucose was measured at fasting and 15, 30, 60, 90, and 120 minutes and insulin were measured at fasting and 15, 30, 60, 90, 120, and 180 min post-test meal consumption. Glucose and insulin incremental area under the curve (iAUC) from fasting to 120 minutes were calculated. SBY antioxidant capacity was measured and its plasma total antioxidant capacity response was measured at 0, 45, 90, and 180 min post-ingestion. Phenolic compounds and their metabolites were detected in SBP, SBY, plasma and urine. Participants ingesting SBY (low GI) and yogurt showed lower glucose and insulin responses after 60 min than those ingesting white bread ($P < 0.05$). Peak plasma glucose and insulin concentrations were reached within 30 and 60 minutes, and within 15 and 60 minutes for all foods, respectively. Both the glucose and insulin iAUC was significantly lower for SBY and yogurt as compared to white bread ($P < 0.05$). Although not statistically significant, a higher plasma antioxidant concentration was observed in participants ingesting SBY compared to those ingesting plain yogurt. This was most likely attributable to the SBP phenolic content. Drawing on these findings, SBY is a potential low GI functional food, loaded with antioxidants from SBP.

6.2 Introduction

Berries are considered among the healthiest foods due to their nutritional components which include vitamins, fibre, and bioactives. The main bioactives in berries are the polyphenols or the phenolic compounds. The latter can be further categorized as anthocyanins, phenolic acids, flavonols, flavan-3-ols, and others (Lachowicz et al, 2017). Accumulating evidence suggests that long term intake of plant polyphenols (fruits and vegetables) can have an impact on the development of specific chronic diseases. These chronic diseases include cancers, cardiovascular diseases, type 2 diabetes mellitus, and impaired cognitive function (Kwon et al, 2007; Alzaid et al, 2013; Rio et al, 2013). In particular, high consumption of berries rich in anthocyanins has been associated with a reduced risk of type 2 diabetes mellitus (Carmen and Visioli, 2017). Impaired glucose tolerance, prediabetes, and diabetes are global health concerns. New prevention and treatment strategies are being sought and these include dietary management through the use of functional foods.

Native to the Canadian culture is the small fruit called the Saskatoon berry (Saskatoons), or serviceberries (Mazza et al, 2005). Recent reports provide evidence that Saskatoon berries are an excellent source of numerous bioactive phenolic compounds (Dudonné et al, 2015; Lavola et al, 2012). Anthocyanins are the major functional components in Saskatoon berries (Mazza et al, 2008). Based on their quantity in the fresh fruit, phenolic compounds of Saskatoon berries belong to four major families. These include anthocyanins, flavonols, flavan-3-ols, and phenolic acids (Lachowicz et al, 2017). The main two anthocyanins quantitatively and qualitatively are cyanidin 3-galactoside (approximately 61% of total anthocyanin content) and cyanidin 3-glucoside, which comprises about 21% of the total anthocyanin content (Mazza et al, 2008; Lachowicz et al, 2017). Concerning absolute concentration, a range of 25 to 179 mg of

anthocyanins/100 grams of berries was reported previously (Mazza et al, 2008). Recently, the Canadian cultivar “Smoky” has been reported to contain 1562.06 mg anthocyanins/100g dry matter (Lachowicz et al, 2017). Lavola *et al* (2012) reported that an anthocyanin content of as much as 382 mg/100 g of fresh weight is amongst the highest amounts reported in cultivated berries.

In vitro evidence suggests that Saskatoon berries have demonstrated antioxidant activity (Hu et al, 2005). Other evidence from *in vitro* studies with berries suggests that anthocyanins do not only contribute to the observed antioxidant activity, but also, may play a role in mitigating the risk of diseases associated with oxidative stress (Dudonné et al, 2015; Juríková et al, 2013). Dietary polyphenols are known to affect many metabolic processes including intestinal glucose absorption. The widely distributed GLUT2 glucose transporter modulates glucose absorption in the presence of plant polyphenols (Kwon et al, 2007; Alzaid et al, 2013).

The glycemic index (GI) values for some of the common berries have been reported (Foster-Powell et al, 2002), and in the fruit closest in composition to Saskatoon berry, blueberry (Mazza, 2008). The latter has a GI value within the range of 47-59 (medium GI) (Muraki et al, 2013). However, Saskatoon berry fruit GI has never been reported. The short harvest period for fresh Saskatoon berries in Western Canada limits the consumption of fresh fruit. To extend access to the nutrients and bioactives found in Saskatoon berries, a freeze-dried Saskatoon berry powder (SBP) has been developed. When added to other foods, including yogurt, the potential exists to position a functional food that may have positive effects in reducing risks associated with dysregulation of glycemic responses. To this end, a test for the functionality of SBY was conducted in a group of healthy participants. The objectives of this study were: 1) To determine the effect of adding SBP to yogurt on the antioxidant capacity of the test food (SBY), 2) To

measure the GI of the test food (SBY), 3) To examine the effect of the test food (SBY) on post-prandial plasma glucose, insulin, and total antioxidant capacity, and 4) To examine the phenolic compounds in SBP and SBY and their respective metabolites in plasma and urine of participants consuming these foods, in order to determine their order of appearance.

6.3 Materials and Methods

6.3.1 Test food development

SBP was prepared by the Food Development Centre, Manitoba, Canada. Briefly, fresh ripe Saskatoon berries (Smoky variety) were harvested and stored at -20°C, then the frozen fruits were freeze dried to produce the powder. At the dairy plant at the University of Manitoba, the plain yogurt was prepared using vitamin D-fortified Medallion® skim milk powder, vitamin D-fortified Lucerne® 1% partly skimmed milk, and yogurt starter culture (YO-MIX 495 LYO 100 DCU yogurt cultures). The starter culture was purchased from Danisco Cultures, composed of 2 species: *Streptococcus thermophiles* and *Lactobacillus delbrueckii* subspecies (*bulgaricus*). SBY was prepared by adding SBP to the freshly prepared plain yogurt. During the production process, the maximum temperature the yogurt reached was 45°C. Forty grams of SBP were added to 300 ml of the yogurt. Proximate analysis was done on the SBY and plain yogurt to determine the serving that would provide 25 grams of digestible carbohydrates (CHO). Based on the proximate analyses results (Table 6.1), table sugar was added to plain yogurt, to make a serving that provides 25 grams CHO. Plain yogurt was portioned in cups of (approximately 150 ml) 162.5 grams and SBY was portioned in cups of (approximately 150 ml) 142.5 grams, so that each serving contained 25 grams of carbohydrates. The yogurts were stored frozen until later use.

Vitamin D and calcium were estimated based on the information found on the food labels and from food composition tables (United States Department of Agriculture Food Composition Database, 2016).

6.3.2 Test food composition

Table 6.1: SBY (test food), yogurt, and white bread nutrient composition

<i>Composition</i>	<i>White bread</i>	<i>Yogurt</i>	<i>SBY</i>
<i>Serving (g)</i>	79.1	162.5	142.5
<i>Carbohydrate (g)</i>	25	25	25
<i>Carbohydrate %</i>	31.6	15.4	17.5
<i>Protein (g)</i>	5.1	7.0	7.0
<i>Protein %</i>	6.4	4.3	4.9
<i>Fat (g)</i>	2.6	0.9	0.9
<i>Fat %</i>	3.3	0.5	0.6
<i>Fibers (g)</i>	0.0	0.0	8.8
<i>Fibers %</i>	0.0	0.0	6.2
<i>Energy</i>	201.6	112.3	114.0
<i>Calcium</i>	NA [*]	253.37	318.52
<i>Vitamin D</i>	NA [*]	94.78	83.12

^{*}NA: not available.

6.3.3 Test food pH measurement

SBY and plain yogurt samples (3 samples) were removed from frozen storage (-20°C) and kept in the refrigerator overnight to allow for sample thawing before pH measurement.

Thawed samples were mixed thoroughly, and 3 replicates of each of the samples were poured

into smaller cups in preparation for pH reading (Oakton 35624-35, Oakton Instruments, Vernon Hills). Measurements were done in triplicate.

6.3.4 Test food antioxidant capacity:

6.3.4.1 Phenolic compounds extraction

SBP and SBY samples were extracted by an ultrasound-assisted extraction method, according to (Oszmianski et al, 2015) with a slight modification done for the SBY extraction. 0.50 g of SBP and 1.05 g of the SBY samples (3 samples, 3 replicates each) were extracted using 25 ml of 50% methanol (1% acetic acid) solvent, under sonication for 2 sessions of 20 minutes. Then, the extract was centrifuged at 13,000x g for 10 minutes. The supernatant was stored refrigerated (4°C) no longer than 24 hours for use in other analyses.

6.3.4.2 2,2-diphenyl-1-picrylhydrazyl (DPPH)

1.5 ml of DPPH/methanol was mixed with 125µL SBP, SBY, plain yogurt, or water (control) in triplicate. The mixtures were vortexed for 30 seconds and allowed to stand at room temperature in the dark, for 10 minutes. The DPPH absorbance was measured (triplicate measurements) spectrophotometrically at 516 nm, readings were recorded and compared to water (control). The inhibition % was calculated according to the formula:

$$\text{Inhibition\%} = \frac{\text{A516 control} - \text{A516 extract} * 100}{\text{A516 control}}$$

DPPH absorbance represents its ability as a radical scavenger (trap). The radical scavenging ability of SBP and SBY extracts was measured at a 2,2-diphenyl-1-picrylhydrazyl (DPPH) concentration of 60 mmol/L in methanol, as described by Shori and Baba (2013) with slight modifications (in this experiment methanol was used instead of ethanol). The chemical reaction rate reduction upon the addition of DPPH represents the radical nature of that reaction (the scavenging capacity of SBP and SBY). DPPH occurs purple at around 516-520 nm, and turns into colorless or pale yellow when neutralized.

6.3.4.3 Oxiselect ferric reducing antioxidant power (FRAP)

Antioxidant capacity of SBP, SBY, and plain yogurt extracts was measured using Oxiselect ferric reducing antioxidant power (FRAP) assay kit (STA-859, available through Cell Biolabs). The FRAP kit is a quantitative assay for measuring the antioxidant potential in a sample. The antioxidants within the sample reduced the ferric iron to its ferrous form, which corresponded to a change in the color to a dark blue. The absorbance was measured (triplicate measurements) through an OMEGA reader at 600 nm. Antioxidant potential of samples was determined by comparing the absorbance to the iron standard.

6.3.5 Test food phenolic compounds:

6.3.5.1 Food extract preparation

LC-QTOF-MS samples: 5 samples of SBP (150 mg) of previously freeze-dried fresh Saskatoon berries, 5 samples of plain yogurt (1.125 ml), and 5 samples of SBY (1.125 ml) were dissolved in (3:2, v/v) methanol: water. Samples were vortexed for 10 seconds at 3000 rpm, and centrifuged at 10,000 rpm for 30 minutes using an IEC MicroMax centrifuge. Each supernatant

was transferred into clean Eppendorf tubes and dried under nitrogen flow for up to 8 hours until completely dry. The residue was reconstituted in 1 ml (4:1, v/v) water: acetonitrile, vortexed until fully reconstituted, then centrifuged at 10000 rpm for 10 minutes. Samples were kept in the fridge at 4°C until they were analyzed with a QTOF-MS Agilent Technologies 6538 accurate-mass quadrupole time of flight mass spectrometer (Agilent Technologies, Santa Clara, USA).

NMR samples: 3 samples of SBP (150 mg) of previously freeze-dried fresh Saskatoon berries, 5 samples of plain yogurt (1.125 ml), and 5 samples of SBY (1.125 ml) were dissolved in (3:2, v/v) methanol: water. Samples were vortexed for 10 seconds at 3000 rpm, and centrifuged at 10,000 rpm for 30 minutes using an IEC MicroMax centrifuge. Each supernatant was transferred into clean Eppendorf tubes and dried under nitrogen flow for up to 8 hours until completely dry. The residue was reconstituted in 1 ml (4:1, v/v) water: acetonitrile, vortexed until all residue is reconstituted, then centrifuged at 10000 rpm for 10 minutes. 200 µl of sample was added to 350µl of (4:1, v/v) D₂O: deuterated acetonitrile and transferred into 5mm NMR tubes (Bruker). Tubes were covered and inserted into the ¹H NMR device, ¹H NMR spectra were recorded with a BrukerTM ASCEND 600 MHz NMR magnet system operating at a proton frequency of 600.13 MHz.

6.3.5.2 LC-QTOF-MS analysis and data processing

LC-QTOF-MS analysis: A search for all previously identified phenolic compounds and their metabolites was performed, looking into peer-reviewed articles published between 2010 and May of 2017. The search involved all articles appearing on PubMed with at least one of the following keywords: phenolic compounds, phenolics, bioactives, berry, berries, Saskatoon berry, phenolic compounds metabolites, antioxidant, plasma, and urine. The search resulted in 45

articles and 351 phenolic compounds/ their metabolites, and the chemical formulas were obtained through METLIN metabolite database (<https://metlin.scripps.edu/>) and PubChem database (<https://pubchem.ncbi.nlm.nih.gov/search/>). Mass spectrometry was done using an Agilent Technologies 6538 accurate-mass quadrupole time of flight mass spectrometer, equipped with a dual electrospray ionization source (ESI) both in negative and positive modes. A targeted analytical approach (351 phenolic compounds/ metabolites) was utilized to identify all potential compounds/ metabolites that can be detected in the samples. The metabolite datasets obtained for the food extract were analyzed through MHQ (Agilent MassHunter Qualitative software tool) as described in detail previously (Hanson, 2018).

6.3.6 Study design and protocol

A single-site, randomized, and controlled interventional post-prandial design, with 4 intervention visits and 1-2 screening visits was conducted during 2015 and 2016. The study protocol was approved by the Research Ethics Board (REB) at the University of Manitoba and the St. Boniface Hospital Research Review Committee. The study was registered on ClinicalTrials.gov (<https://clinicaltrials.gov>), and given the code (NCT02571114) (Appendix A).

6.3.6.1 Participants

12 healthy participants (8 males and 4 females) were recruited at the St. Boniface hospital (I. H. Asper Clinical Research Institute) through local advertisement. Recruitment started in March (2015) and ended by June (2016). The inclusion criteria were: healthy individuals aged 18-35 years, non-smoking, with no history of: diabetes, metabolic syndrome, or any other chronic metabolic diseases. Male and female (not pregnant or lactating) volunteers were

screened for being generally healthy, assessed by a screening questionnaire, body mass index (normal to overweight), blood pressure, standard hematology, and liver and kidney functions. The participants have been on stable regimen for any nutritional and herbal supplements over the past 3 months prior to the study period (almost 6 weeks). All participants (n=12) signed a written consent form and completed the study, which included not consuming Saskatoon berries or any of its products while participating in the study. The participants' demographics are shown in Table 6.2.

Table 6.2: Baseline characteristics of study participants

<i>Characteristics (n=12)</i>	<i>Average</i>	<i>Minimum</i>	<i>Maximum</i>
<i>Males (n=8)</i>			
<i>Females (n=4)</i>			
<i>Age (years) Males</i>	27.9	21.0	34.0
<i>Age (years) Females</i>	25.3	20.0	32.0
<i>Weight (kg) Males</i>	79.6	58.0	99.0
<i>Weight (kg) Females</i>	64.1	59.5	69.0
<i>BMI^a (kg/m²) Males</i>	25.41	20.3	29.7
<i>BMI (kg/m²) Females</i>	25.2	23.5	28.7
<i>Blood pressure (mmHg)^b Males</i>	113/78	97/67	127/86
<i>Blood pressure (mmHg) Females</i>	105/70	91/57	111/81
<i>HbA1c^c Males</i>	5.2	4.6	5.5
<i>HbA1c Females</i>	5.3	5.2	5.3

^a BMI: Body mass index was calculated using weight in kilograms divided by height in meters squared.

^b mmHg: Millimeter mercury.

^c HbA1c: Glycated hemoglobin A1c.

6.3.6.2 Blood sample collection

Participants were requested to fast for 12-16 hours before the day of each treatment visit. During each visit, 10-20 ml of venous blood were collected before food consumption (time = 0 minutes) and 3 hours after the participants have consumed the test food (time = 180 minutes). During the 3 hours, capillary blood was collected by finger prick at time points: 0, 15, 30, 60, 90, and 120 minutes from the start of the test food consumption. Plasma was obtained by centrifugation according to the procedure described in Edel et al. (2015). Plasma were separated into small aliquots and stored in the -80 °C immediately until being used for analyses.

6.3.6.3 Postprandial glycemic and insulinemic responses

Plasma glucose concentrations were measured from finger prick blood collections, directly using a glucometer, and measures were done at time points: 0, 15, 30, 60, 90, and 120 minutes from the start of the study visit. Plasma insulin concentrations were measured from finger prick blood collections, using an ultrasensitive insulin ELISA kit (ALPCO, 80-INSHUU-E10), and measures were done at time points: 0, 15, 30, 60, 90, 120, and 180 minutes from the start of the study visit. All samples were analyzed in duplicate for insulin, with a CV% of no more than 23%. The incremental area under the curve (iAUC) from 0 to 120 minutes for glucose and insulin was calculated as previously described (Brouns et al, 2005). The glycemic index (GI) for yogurt and SBY was calculated by dividing iAUC of the food by the iAUC of the white bread (average of 2 bread visits), and multiplied by 100.

6.3.6.4 Postprandial antioxidant responses (TAC and FRAP)

Plasma total antioxidant capacity was measured from venous blood collections, using 2 commercially available assay kits, including the total antioxidant capacity assay kit (MAK187, available through Sigma Aldrich), and the Oxiselect ferric reducing antioxidant power (FRAP) assay kit (STA-859, available through Cell Biolabs). Measures were taken at time points: 0, 45, 90, and 180 minutes from the start of the study visit. All samples were analyzed in duplicate for both TAC and FRAP, with a CV% of no more than 10%.

6.3.6.5 Postprandial phenolic compounds in plasma and urine:

6.3.6.6 Sample extraction

10-20 mL of venous blood was collected before the test food consumption from all study participants (n=12). After the participants have consumed the test food, venous blood was collected from a subsample (n=4): 45 minutes, 90 minutes, and 180 minutes post-ingestion. Plasma were obtained by centrifugation according to the procedure described in Edel et al. (2015). Plasma samples were separated into small aliquots, appropriately labelled and stored in the -80 °C immediately until being used for analyses. For extraction analysis, samples were thawed on ice for 20 minutes, vortexed and 100µl aliquots (duplicates) were added to 200µl acetonitrile, and vortexed vigorously for 30 seconds. Mixtures were centrifuged at 10000xg for 10 minutes, and the supernatants were dried to completeness under gentle nitrogen flow, then stored at -20 °C until further analysis. On the day of analysis, samples were reconstituted in 100µl of (4:1, v/v) acetonitrile: water and 10µl D-norvaline (standard) was added. Urine samples were collected using toilet hat specimen collectors before food consumption and 3 hours after the

participants had consumed the test food (n=12). Urine was separated into smaller aliquots of 2 and 5 ml, labelled and kept in the -80 °C until being used for further analyses. For extraction analysis, urine samples were thawed on ice, 250µl aliquots (duplicates) were vortexed with 10µl D-norvaline (standard) for 10 seconds, and samples were then centrifuged for 10 minutes at 10000xg. Supernatants were mixed with 500µl of acetonitrile and vortexed for 10 seconds and kept in the -20 °C for 30 minutes for quenching, after that samples were centrifuged at 10000xg for 20 minutes (room temperature), dried under nitrogen flow and reconstituted with 200µl of (4:1, v/v) water: acetonitrile.

6.3.7 Statistical analyses

To compare the difference of the mean values (test food) for pH, DPPH radical scavenging capacity, FRAP antioxidant capacity, GI and GL, a single factor ANOVA was performed to compare the means of the parameters, and data is presented as means $\pm$ SD. To assess the postprandial effects of the test food on glucose, insulin, and antioxidant capacity, a one-way ANOVA with repeated measurements was performed for glucose and insulin, and the data is presented as means $\pm$ SEM. A paired t-test procedure was performed for antioxidant capacity (TAC and FRAP), and data is presented as means $\pm$ SEM.

The postprandial glucose and insulin concentrations of the two white bread visits were averaged and used as a reference. SPSS (version 23 for Mac) statistical analysis software tool was used to perform all analyses.

6.4 Results

6.4.1 Test food (SBY) pH

Berry fruits and yogurt are acidic foods (Aloglu and Öner, 2011; Zatylny et al, 2005), and adding SBP to yogurt further lowered its pH from 4.15 ± 0.01 to 3.89 ± 0.04 (Table 6.3). However, the reduction was not statistically significant.

Table 6.3: SBY (test food) and yogurt pH

<i>Food</i>	<i>pH $\pm$ SD</i>
<i>Yogurt</i>	4.15 ± 0.01
<i>Saskatoon berry yogurt</i>	3.89 ± 0.04

6.4.2 Test food antioxidant capacity

DPPH is a simple and rapid experiment, which measures the % inhibition of antioxidant molecules, and is widely used with food samples (Hangun-Balkir and McKenney, 2012). A dose of 0.50 gram of the SBP alone showed significantly lower DPPH inhibition % than the same amount of powder when added to yogurt (SBY) (Table 6.4).

To evaluate dose response effects of SBP either alone or as SBY on the total antioxidant capacity, 3 dosage levels were evaluated using the FRAP method. Samples of SBP were tested as such and as SBY, assuming that a different amount of the SBP would have a different FRAP, and the results confirm these assumptions. The FRAP value (the antioxidant power) of SBP (0.05 grams) is significantly higher than that of the SBY.

Table 6.4: SBY (test food) and Saskatoon berry powder antioxidant capacity (DPPH inhibition % and FRAP)

<i>Sample</i>	<i>Amount (in gram or milliliter)</i>	<i>Inhibition % of DPPH ± SD or FRAP ± SD</i>
	<i>Amount (g/ml)</i>	<i>Inhibition % of DPPH ± SD</i>
<i>Saskatoon berry powder</i>	0.50 g	90.87 ± 0.07 ^b
<i>Saskatoon berry yogurt</i> [#]	1.05 ml (0.50 g SBP)	91.27 ± 0.06 ^a
	5.00 ml (2.38 g SBP)	89.81 ± 0.14 ^c
	10.00 ml (4.75 g SBP)	88.5 ± 0.14 ^d
<i>Plain yogurt</i>	1.05 ml	ND [*]
	5.00 ml	ND [*]
	10.00 ml	ND [*]
	<i>Amount (g/ml)</i>	<i>FRAP ± SD (μM)</i>
<i>Saskatoon berry powder</i>	0.50 g	488 ± 48.6 ^b
	4.75 g	587 ± 5.1 ^a
<i>Saskatoon berry yogurt</i>	1.05 ml (0.50 g SBP)	263.7 ± 29.3 ^c
	10 ml (4.75 g SBP)	244.1 ± 10.9 ^d
<i>Plain yogurt</i>	1.05 ml	138 ± 31.87 ^e
	10 ml	155.6 ± 35.06 ^c

^{*}ND: not determined.

[#]The amount of SBY and its control (plain yogurt) was based off the SBP sample size (0.5 g SBP). 0.5 g of SBP is dissolved in a volume of yogurt equivalent to 1.05 ml. An amount that is 5 times and 10 times the SBY (originally containing 0.5 g SBP) was then tested to evaluate the effect of the potential difference in the physiological effect of SBY once metabolised.

^aMeans with different superscript alphabets are significantly different (a-e), P< 0.05.

6.4.3 Test food phenolic compounds

Proton NMR spectroscopy was used to detect the phenolic compounds in SBP, plain yogurt, and the test food (SBY). The spectra show aromatic compounds represented by caffeic acid derivatives detected within a range of 5.6-8 ppm (Figure 6.1) the top view spectra. A comparison between plain yogurt, SBP and SBY (Figure 6.2) shows that the aromatic region is retained after the addition of the freeze-dried SBP to the plain yogurt, and storage of SBY for more than 6 months in the -20°C.

Figure 6.1: Representative ^1H NMR spectrum of SBP. Spectrum showing the presence of sugars and malic acid as major components (δ 2.5 = δ 5.5) region, and the phenolic compounds (caffeic acid derivatives) (δ 5.8 = δ 7.8) region are shown with signal amplification. Spectrum obtained on a 600 MHz NMR spectrometer

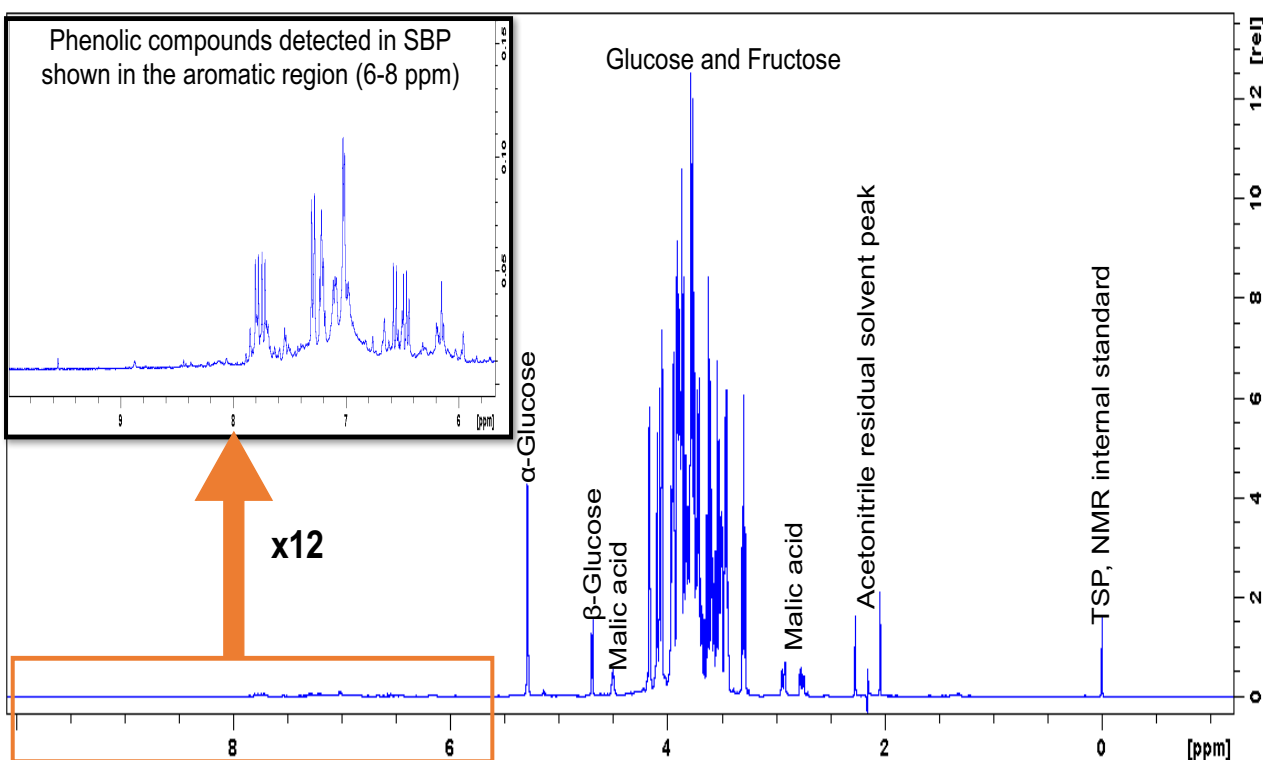
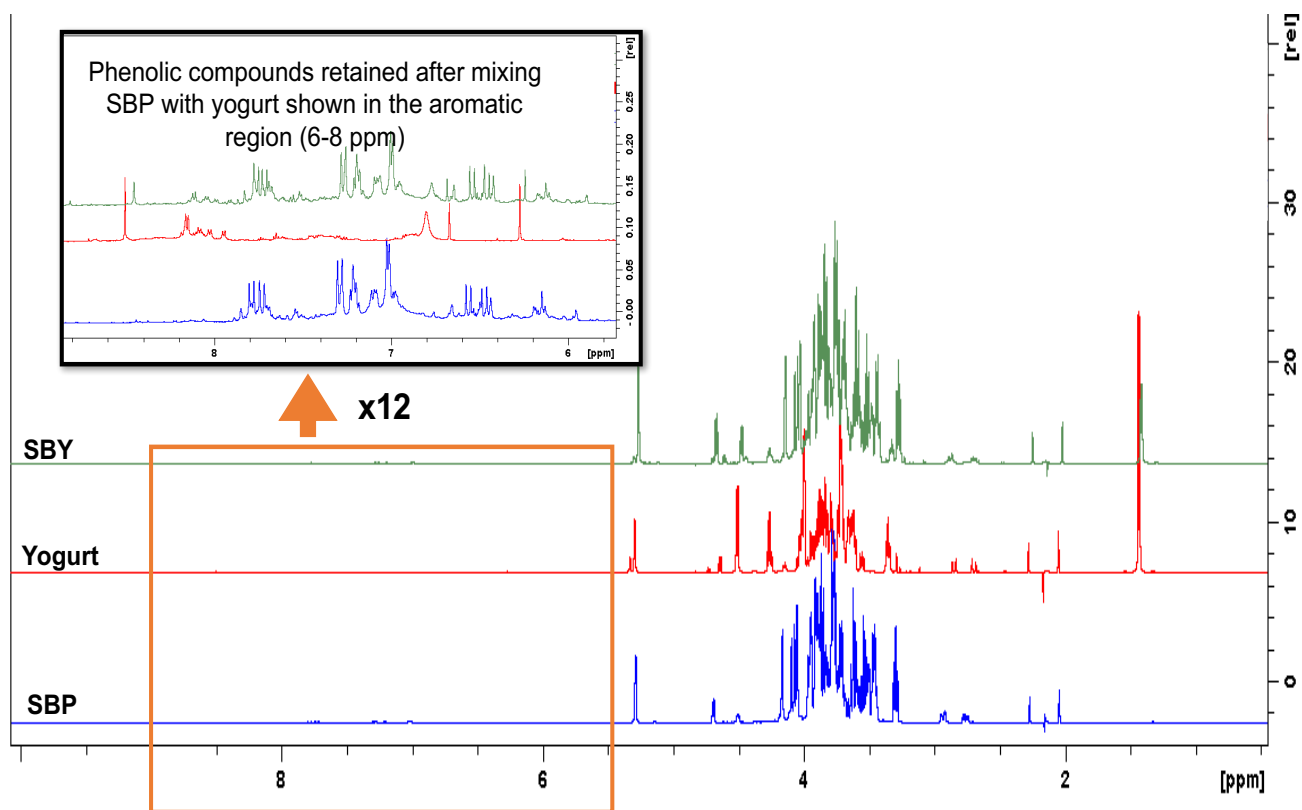


Figure 6.2: Comparison of ^1H NMR spectra of SBP (blue), yogurt (red) and SBY (green) showing the phenolic compounds (caffeic acid derivatives) region retained in SBY (test food). The phenolic compounds (δ 5.8 - δ 7.8) regions are shown with signal amplification. Spectra obtained on a 600 MHz NMR spectrometer



6.4.4 Glycemic index and glycemic load

The SBY exhibited a GI value of 36.4 and plain yogurt a GI of 44.8, and each is classified as a low GI food (Table 6.5). Although plain yogurt is normally reported to have a low GI, in this study table sugar (sucrose) was added to the yogurt to adjust for the total carbohydrate content to 25 grams/serving. Both SBY and plain yogurt classify as low GL.

Table 6.5: SBY (test food), yogurt, and white bread GI % and GL (mean values $\pm$ standard error)

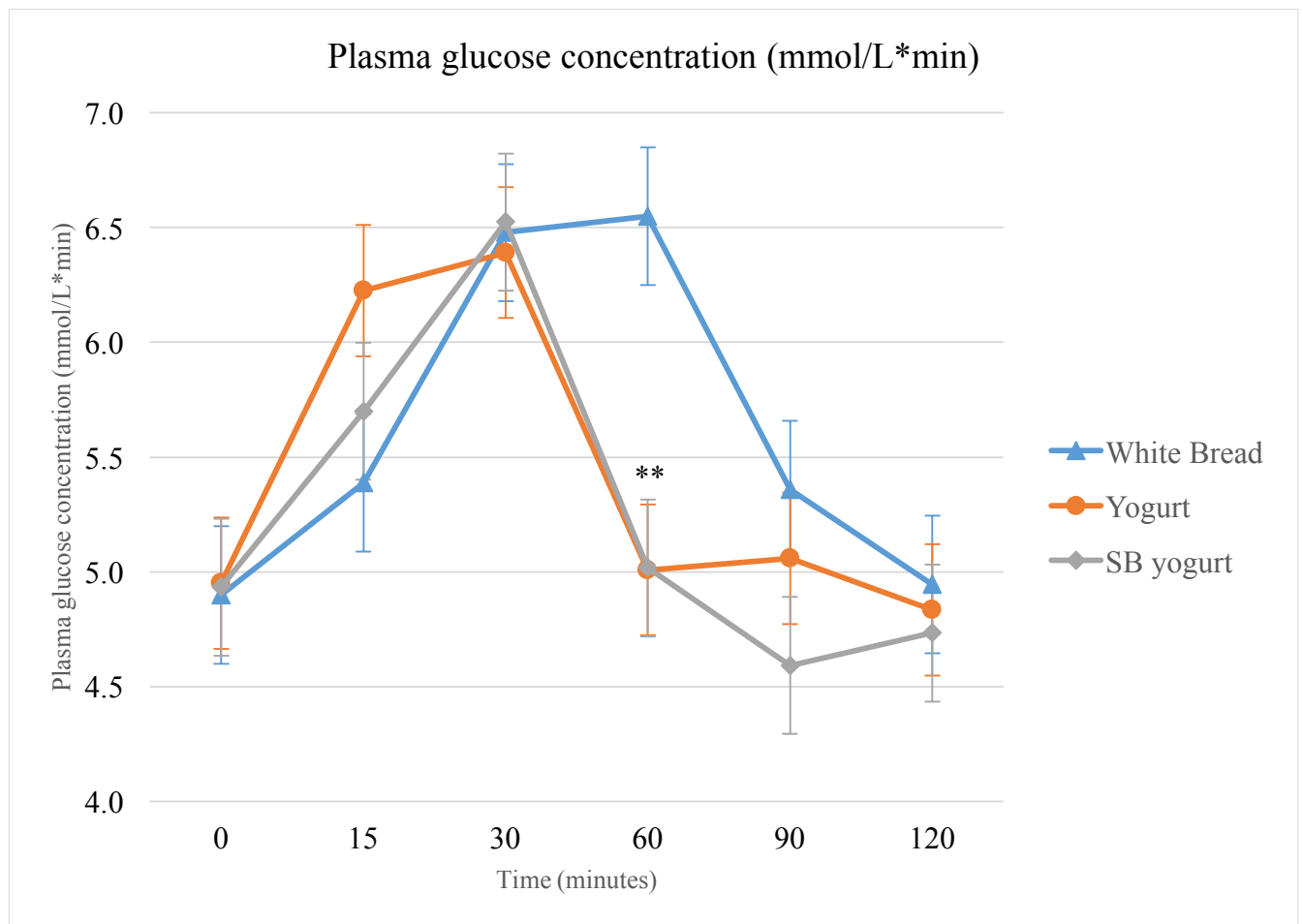
	<i>White bread</i>	<i>Yogurt</i>	<i>SBY</i>
<i>GI</i>	100	44.8	36.4
<i>Classification</i>	High	Low	Low
<i>GL*</i>	28	11.2	9.1
<i>Classification</i>	High	Medium	Low

*GL values were calculated from food composition (Table 6.1) and GI values.

6.4.5 Glycemic response (n=12)

The fasting plasma glucose concentrations of the 12 participants were not statistically different for each of the 4 visits. There was no alteration in glycemic response between all groups at 30 min. However, after 60 minutes the glycemic response was lower in participants who ingested SBY or plain yogurt compared to participants that ingested white bread ($P < 0.05$) (Figure 6.3). Peak plasma glucose levels were reached within 30 and 60 minutes post-ingestion for all the foods (SBY, plain yogurt, and white bread).

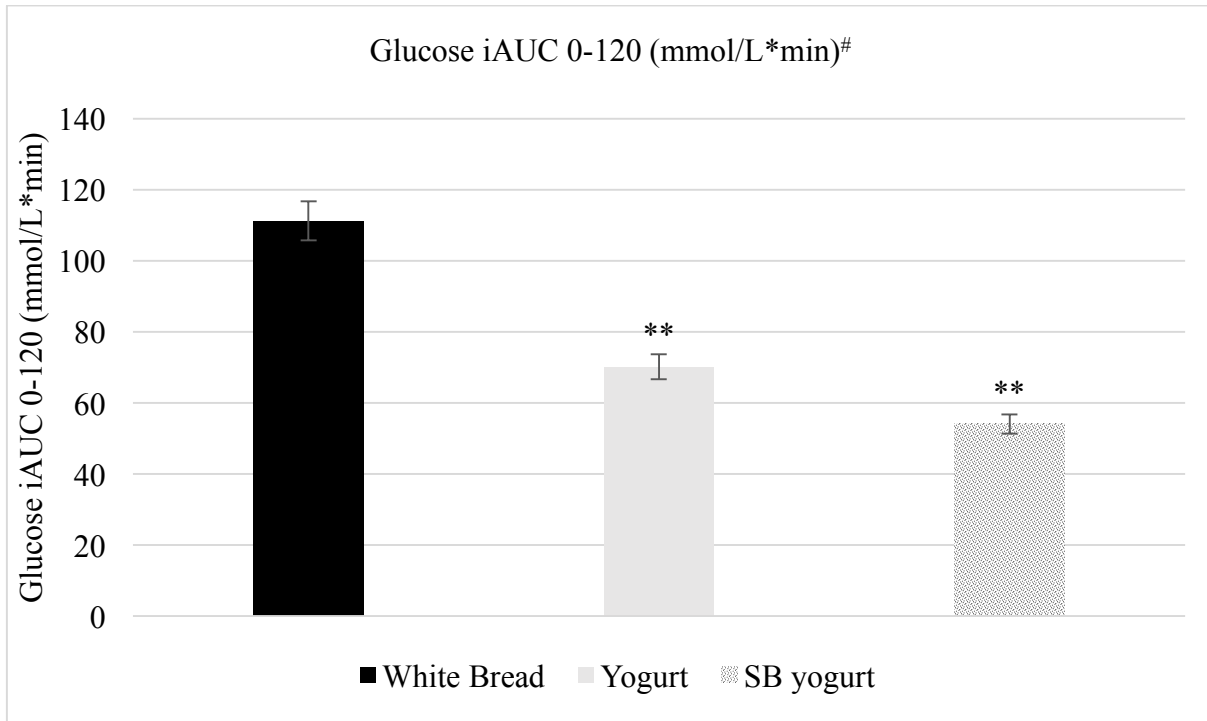
Figure 6.3: Postprandial plasma glucose responses (mmol/L*min) after consuming SBY (test food) (◆), yogurt (●), and white bread (▲), data shown as mean values $\pm$ SEM, (** P<0.05)



** Double asterisk indicate significant difference between the treatments (SB yogurt and yogurt) and the control (P<0.05).

The glucose iAUC for both the SBY and the plain yogurt was significantly lower than that of the white bread (P<0.05), as shown in Figure 6.4.

Figure 6.4: Glucose iAUC (mmol/L*min) after consuming SBY (test food), yogurt, and white bread, data shown as mean values $\pm$ SEM, (** P<0.05)



[#]mmol/L*min: millimole/ liter*minute.

** Double asterisk indicate significant difference between the treatments (SB yogurt and yogurt) and the control (P<0.05).

6.4.6 Insulinemic response (n=10)

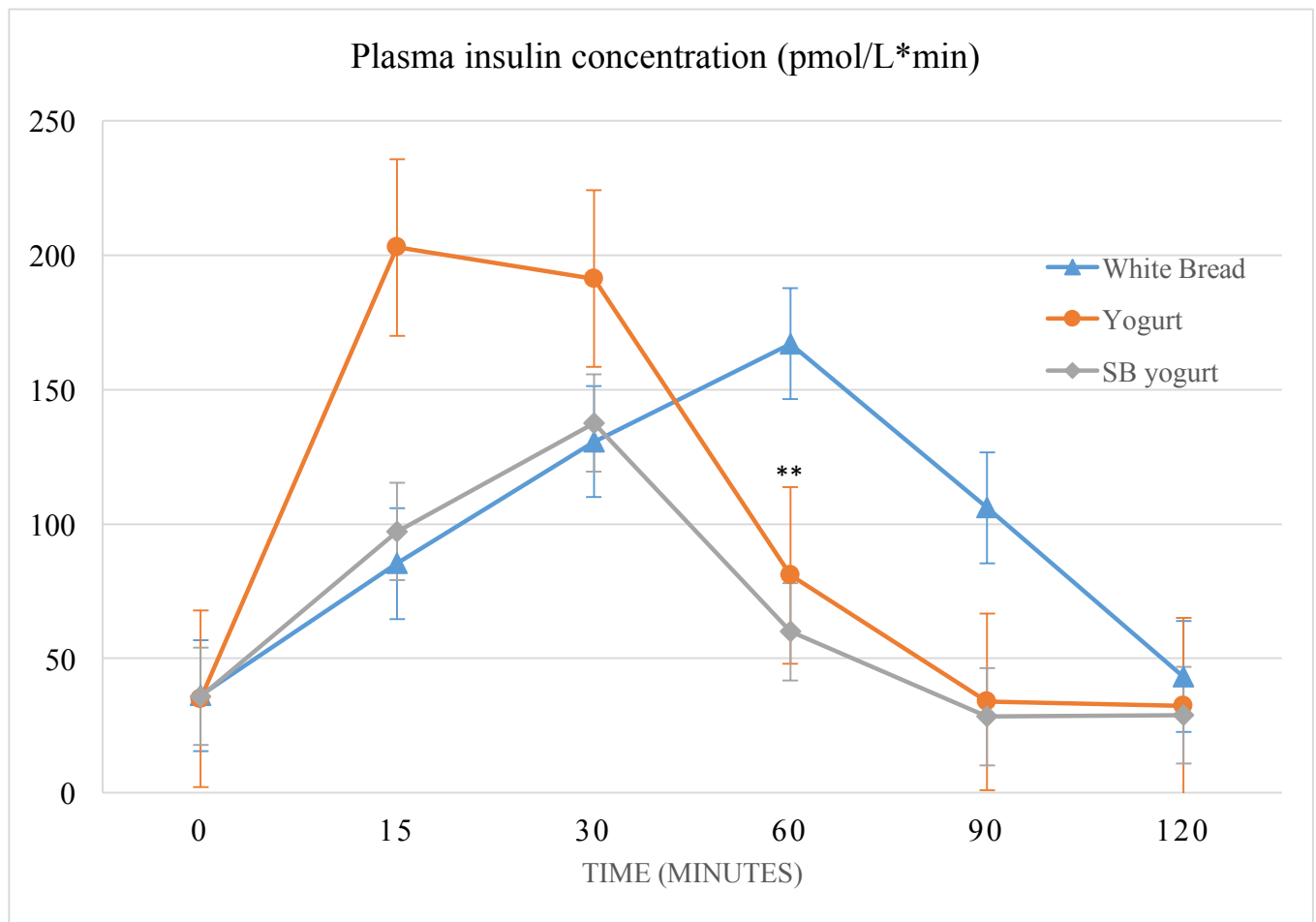
Initially insulin data was collected from all 12 participants. However, data from 10 out of 12 participants were considered for statistical analysis. The other 2 participants' data were excluded from the analysis due to one or more of the following: (1) Missing samples: samples were collected through finger prick blood, and in some cases no more blood was drawn after performing 3 finger pricks, and/or (2) The ELISA kit used to analyze the insulin was an ultrasensitive kit. The total sample collected was 50µl or less and the ELISA kit used for analysis requires at least 60µl (30µl each, 60µl for duplicate analysis). Both the sensitivity of the kit (the dilution factor could not be predicted for each participant only with limited sample, and it was highly variable between participants) along with the limited sample size resulted in values that could not be detected by the plate reader (>> range). (3) For some of the successful samples (analyzed), the CV% of the 2 replicates was higher than 25%, in some cases it reached up to 70%, which indicates an error.

For the yogurt treatment, an outlier was found (fasting insulin concentration was more than 2 standard deviations (SD= 55.39 pmol/L) far from the mean (43.22 pmol/L)), so the total number of participants that was considered for statistical analysis was 10 for the white bread and the SBY, and 9 participants for the plain yogurt, as 1 participant's insulin data was excluded from the analysis. Although the excluded insulin concentration represents accurate data collection and measurement, with the relatively small sample size of 10 participants, it is very likely that including this data point will result in very inaccurate or invalid results.

Fasting plasma insulin concentrations of the 10 participants (SBY and white bread) and of the 9 participants (plain yogurt) were not statistically different for each of the 4 visits. After consumption of the SBY, there was no treatment effect when compared to the plain yogurt.

However, 60 minutes of post-ingestion of both SBY and yogurt, plasma insulin concentrations were significantly lower than those in effect of the white bread ($p<0.05$) (Figure 6.5). Peak plasma insulin levels were reached within 15 and 60 minutes post-ingestion for all the foods (SBY, plain yogurt, and white bread).

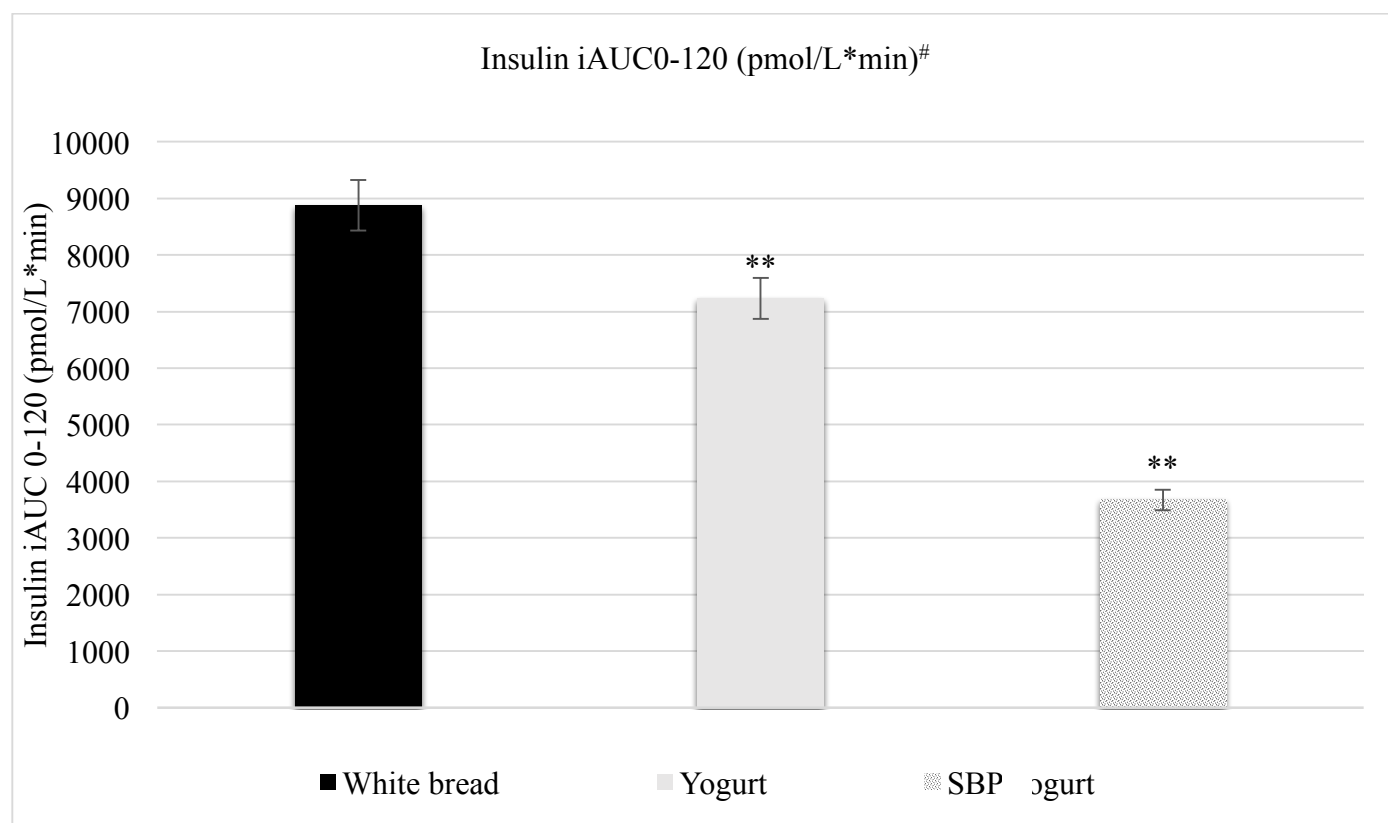
Figure 6.5: Postprandial plasma insulin responses (pmol/L*min) after consuming SBY (test food) (◆), yogurt (●), and white bread (▲), data shown as mean values $\pm$ SEM, (** P<0.05)



** Double asterisk indicate significant difference between the treatments (SB yogurt and yogurt) and the control (P<0.05).

The insulin iAUC for both the SBY and the plain yogurt was significantly lower than that of the white bread (P<0.05), as shown in figure 6.6.

Figure 6.6: Insulin iAUC (pmol/L*min) after consuming SBY (test food), yogurt, and white bread, data shown as mean values $\pm$ SEM, (** P<0.05)



[#]pmol/L*min: picomole/ liter*minute.

** Double asterisk indicate significant difference between the treatments (SB yogurt and yogurt) and the control (P<0.05).

6.4.7 Antioxidant responses (TAC and FRP) (n=4)

Fasting plasma TAC concentrations of the 4 participants were not statistically different for each of the subgroups (each subgroup refers to 4 participants out of the 12), and for the 2 visits (yogurt and SBY). There were no significant differences in plasma TAC concentrations after ingesting SBY in comparison with yogurt, however, the TAC after ingesting SBY was higher after 90 and 180 minutes (figure 6.7). The higher TAC observed following the consumption of plain yogurt (after 45 minutes) might be attributable to an increased (high) baseline TAC in some of the participants (keeping in mind the small sample size of 4 participants), a shift/ deviation from standard in one value would highly affect the other 3. Baseline plasma TAC concentrations of the 4 participants varied widely, potentially due to genetic differences. It is well agreed that analyzing the link between the baseline value and its change overtime is complex, and has always been a matter of inquiry, especially in the case of measuring 2 data points (one baseline value and one overtime change value) (Chiolero et al, 2013).

Fasting plasma FRAP concentrations of the 4 participants were not statistically different for each of the 2 visits (yogurt and SBY). There were no significant differences in plasma FRAP concentrations after ingesting SBY in comparison with yogurt, however, the antioxidant capacity after ingesting SBY was higher after 45, 90, and 180 minutes (figure 6.8).

Figure 6.7: Postprandial plasma TAC response (nmol/ μ L) after consuming SBY (test food) (◆) and yogurt (●) after 45, 90, and 180 minutes

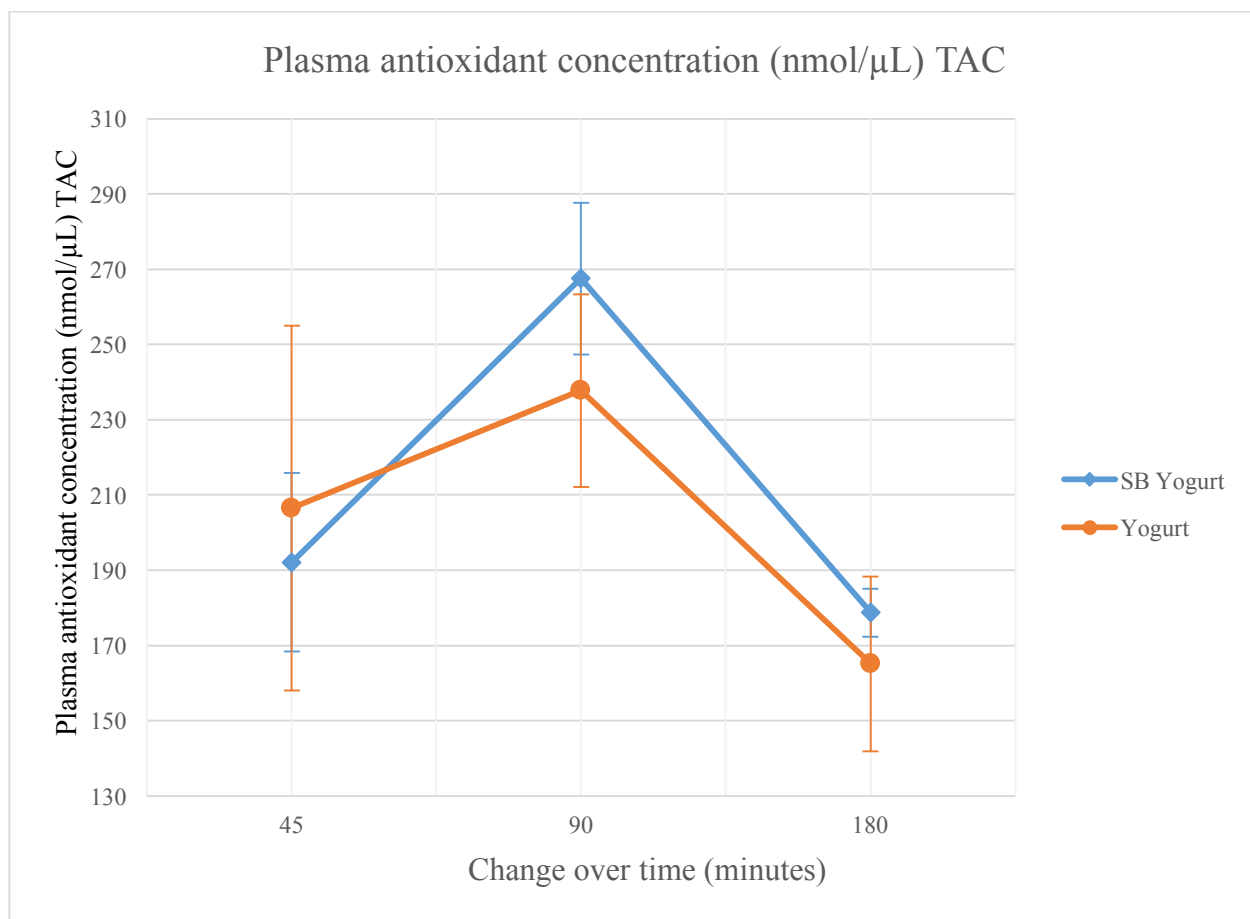
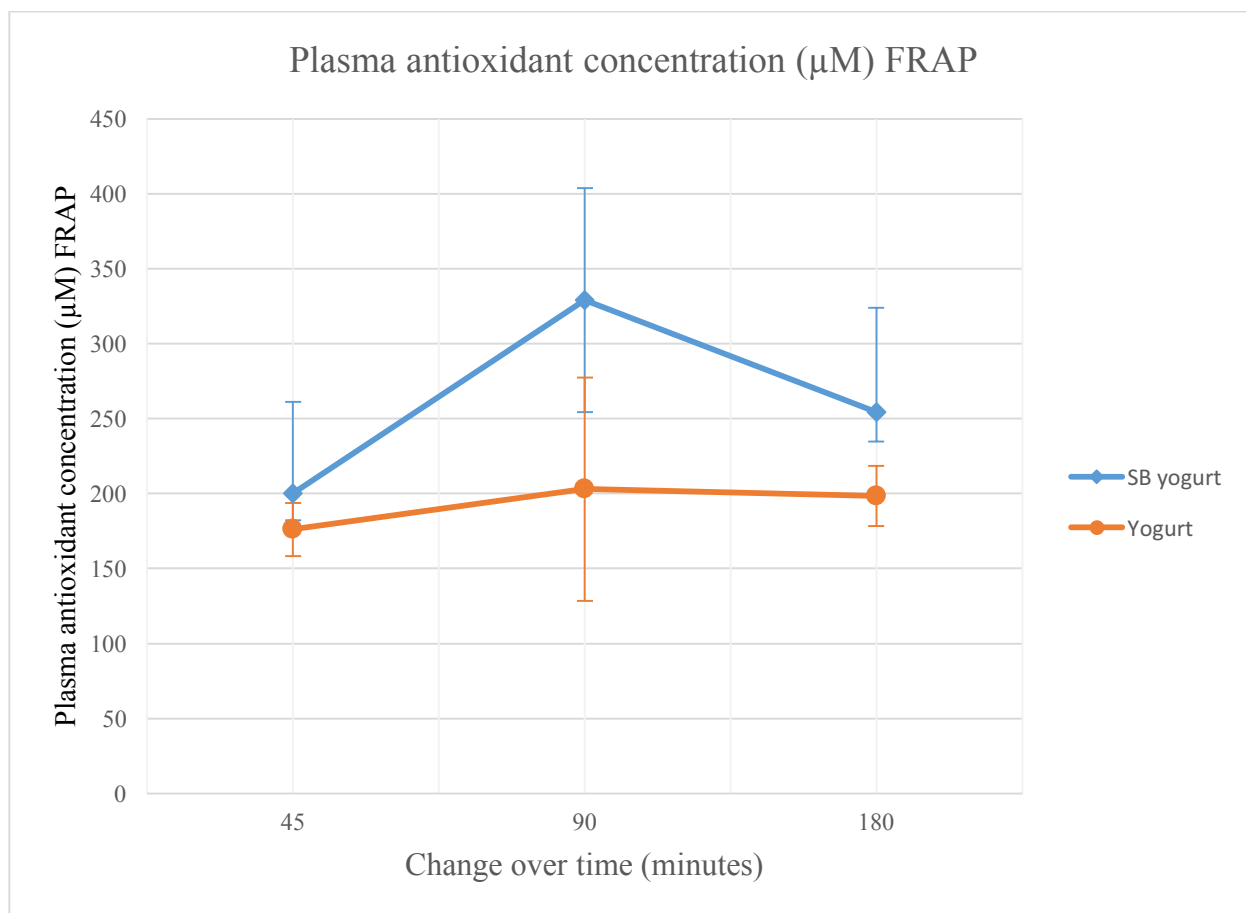


Figure 6.8: Postprandial plasma FRAP response (μM) after consuming SBY (test food) ($\blacklozenge$) and yogurt ($\bullet$) after 45, 90, and 180 minutes



6.4.8 Appearance of phenolics/ metabolites in plasma 45 and 90 minutes post-ingestion (n=4), plasma and urine 180 minutes post-ingestion (n=12)

A list of 351 previously identified phenolics compounds and their relative metabolites or metabolic products was prepared, and all of the compounds' chemical formulas were gathered and used for the analysis. Five samples of each SBP and SBY were analyzed (in triplicate) using an LC-QTOF-MS system, where a targeted approach aimed at detecting all possible phenolic compounds and their metabolic products was used. As a control, 3 samples of each bread and plain yogurt were also analyzed by LC-QTOF-MS using the same approach. None of the phenolic compounds or their metabolites being searched were detected in the bread or in the plain yogurt. Out of the 351 compounds listed previously, 72 compounds were detected in at least one of the food samples or one of the biological samples. Greater than 24 phenolic compounds were detected in SBP, including 2 key anthocyanins of Saskatoon berries, as well as flavonols, flavan-3-ols and others. Twenty-two of the detected compounds were found to be retained in SBY, in addition to a list of 15 other phenolics, including anthocyanins, catechins, flavonols and others. All the 12 participants provided blood and urine samples before (0 minutes) and after (180 minutes) food ingestion. A subgroup of the study participants provided blood samples after 45 (n=4) and 90 minutes (n=4) of food ingestion. None of the phenolic compounds being searched (351) was detected at time point 0 in both plasma and urine, and none was detected after ingestion of the plain yogurt or the bread at any time point post-ingestion. Two phenolic compounds that are retained in SBY (Rhamnetin 3-glucoside and Tricin 7-diglucuronoside) appeared as such in urine after 3 hours. Cyanidin-3-glucoside is the second major anthocyanin in SBY (21% of total anthocyanins) but was not originally detected in SBP, appeared in circulation after 180 minutes. Rutinoside is a flavonol detected in SBY, appeared

after 180 minutes in circulation and in urine. 2',4',5,7-Tetramethoxy-8-methylflavonone, never detected in SBY, appeared in 50% of the participants' circulation after 45 minutes of SBY ingestion, and after 180 minutes in urine. Another metabolites, Epicatechin 5,7,3'-trimethylether, was detected in plasma 90 and 180 minnutes after SBY ingestion, but never in SBY.

Table 6.6: Phenolic compounds and their metabolites detected in plasma after 45 and 90 minutes in a subgroup (n=4), and in plasma and urine after 180 minutes (n=12)

<i>Compound</i>	<i>SBP</i>	<i>SBY</i>	<i>Plasma 45 min</i>	<i>Plasma 90 min</i>	<i>Plasma 180 min</i>	<i>Urine 180 min</i>
<i>Formula</i>	<i>(n=5)</i>	<i>(n=5)</i>	<i>(n=4)</i>	<i>(n=4)</i>	<i>(n=12)</i>	<i>(n=12)</i>
<i>Cobalt-precorrin 5A</i>	5	5	0	0	0	0
<i>C45H52CoN4O16</i>						
<i>Peonidin glucoside</i>	5	5	0	0	0	0
<i>C44H50O24</i>						
<i>Cyanidin galactoside</i>	5	5	0	0	0	0
<i>C43H48O24</i>						
<i>Cyanidin 3-[6''-(4-glucosylferulyl)-sophoroside]-5-glucoside</i>	5	5	0	0	0	0
<i>C49H58O29</i>						
<i>Cyanidin 3-(6''-malyglucoside)</i>	5	5	0	0	0	0
<i>C25H24O15</i>						
<i>Catechin-4-ol 3-O-beta-D-galactopyranoside</i>	5	0	0	0	0	0
<i>C21H24O12</i>						
<i>Quercetin 3-(6''-malonylneohesperidoside)</i>	5	5	0	0	0	0
<i>C30H32O19</i>						
<i>Tricin 7-diglucuronoside</i>	5	5	0	0	0	1
<i>C29H30O19</i>						
<i>Rhamnetin 3-glucoside</i>	5	5	0	0	0	1
<i>C22H22O12</i>						

<i>Rutin</i>	5	5	0	0	0	0
<i>C27H30O16</i>						
<i>Quercitrin</i>	5	5	0	0	0	0
<i>C21H20O11</i>						
<i>Quercetin 3-sambubioside</i>	5	0	0	0	0	0
<i>C26H28O16</i>						
<i>Cyanidi-3,5-O-diglucoside</i>	5	5	0	0	0	0
<i>C27H31O16</i>						
<i>Cyanidin</i>	5	5	0	0	0	0
<i>C15H11O6</i>						
<i>Isorhamnetin 3-glucoside 7-rhamnoglucoside</i>	5	5	0	0	0	0
<i>C34H42O21</i>						
<i>Leucodelphinidin</i>	5	5	0	0	0	0
<i>C15H14O8</i>						
<i>Matteflavoside A</i>	5	5	0	0	0	0
<i>C33H40O18</i>						
<i>Pinoquercetin</i>	5	5	0	0	0	0
<i>C16H12O7</i>						
<i>Epigallocatechin-7-glucuronide</i>	5	5	0	0	0	0
<i>C21H22O13</i>						
<i>Isorhamnetin 3-glucoside 7-rhamnoglucoside</i>	5	5	0	0	0	0
<i>C34H42O21</i>						

<i>Kaempferol 3-[2''-(p-coumaroylglucosyl)rhamnoside]</i> <i>C36H36O17</i>	5	5	0	0	0	0
<i>Cyanidin 3-O-β-D-sambubioside</i> <i>C26H29O15</i>	5	5	0	0	0	0
<i>Carbobenzoxy-L-glutamic acid</i> <i>C13H15NO6</i>	5	5	0	0	0	0
<i>Isorhamnetin 3-glucoside 7-rhamnoglucoside</i> <i>C34H42O21</i>	5	5	0	0	0	0
<i>Procyanidin dimer A type pentoside isomer</i> <i>C35H32O16</i>	0	5	0	0	0	0
<i>Dillenetin 5-glucoside-7-glucuronide</i> <i>C29H32O18</i>	0	5	0	0	0	0
<i>3,4,2',3',4'-Pentahydroxychalcone 4'-O-(2''-O-caffeoyl-6''-O-p-coumaroylglucoside)</i> <i>C39H34O16</i>	0	5	0	0	0	0
<i>O-Methyl epicatechin gallate</i> <i>C23H20O11</i>	0	5	0	0	0	0
<i>4'-Methyl-epigallocatechin</i> <i>C16H16O7</i>	0	5	0	0	0	0
<i>Epigallocatechin 3-O-p-coumarate</i> <i>C24H20O9</i>	0	5	0	0	1	0
<i>Rutinose</i>	0	5	0	0	1	8

<i>C12H22O10</i>						
<i>Eriocitrin</i>	0	5	0	0	0	0
<i>C27H32O15</i>						
<i>Ricinitin</i>	0	5	0	0	0	0
<i>C29H32O17</i>						
<i>Kaempferol</i>	0	5	0	0	0	0
<i>C15H10O6</i>						
<i>Quercetin 3-(2'''-feruloylsophoroside)</i>	0	5	0	0	0	0
<i>C37H38O20</i>						
<i>Luteolin 7-O-(2-apiosyl-6-malonyl)-glucoside</i>	0	5	0	0	0	0
<i>C29H30O18</i>						
<i>Quercetin 3-sambubioside-3'-glucoside</i>	0	5	0	0	0	0
<i>C32H38O21</i>						
<i>Tetramethylquercetin 3-rutinoside</i>	0	5	0	0	0	0
<i>C31H38O16</i>						
<i>ent-Epicatechin-(2alpha->7,4alpha->8)-epicatechin 3-galactoside</i>	0	5	0	0	0	0
<i>C36H34O17</i>						
<i>Procyanidin C1 3,3',3''-tri-O-gallate; [3-O-Galloylepicatechin-(4beta->8)]2-epicatechin-3-O-gallate</i>	0	5	0	0	0	0
<i>C66H50O30</i>						
<i>Cyanidin-3-glucoside</i>	0	5	0	0	1	0

<i>C21H21O11</i>						
<i>2',4',5,7-Tetramethoxy-8-methylflavonone</i>	0	0	2 (50%)	0	0	2
<i>C20H22O6</i>						
<i>Cyanidin 3-O-(6''-acetyl-arabinoside)</i>	0	0	1 (25%)	0	0	0
<i>C22H21O11</i>						
<i>Kaempferol 3-(2'''-(E)-caffeoylglucosyl)-(1>2)-glucoside-7-cellubioside</i>	0	0	1 (25%)	0	0	0
<i>C48H56O29</i>						
<i>4'-Methyl-(-)-epigallocatechin 3'-glucoronide</i>	0	0	3 (75%)	0	0	0
<i>C22H24O13</i>						
<i>Kaempferol 3-(2'''-(E)-caffeoylglucosyl)-(1->2)-glucoside-7-cellobioside</i>	0	0	2 (50%)	0	0	0
<i>C48H56O29</i>						
<i>Guibourtinidol-(4alpha->6)-catechin</i>	0	0	3 (75%)	0	2	0
<i>C30H26O10</i>						
<i>Epicatechin 3-O-(4-O-methylgallate)</i>	0	0	0	2 (50%)	0	0
<i>C23H20O10</i>						
<i>Cyanidin 3-O-(6''-acetyl-arabinoside)</i>	0	0	0	1 (25%)	0	0
<i>C22H21O11</i>						
<i>Kaempferol 3-(2'''-(E)-caffeoylglucosyl)-(1>2)-glucoside-7-cellubioside</i>	0	0	0	1 (25%)	0	0
<i>C48H56O29</i>						

<i>Epicatechin 5,7,3'-trimethylether</i> <i>C18H20O6</i>	0	0	0	1 (25%)	1	0
<i>Cyanidin 3-lathyroside</i> <i>C26H29O15</i>	0	0	0	2 (50%)	0	0
<i>Cyanidin 3-(6'' ferulyl-2''' -sinapylsophorose-5-glucoside</i> <i>C54H59O28</i>	0	0	0	3 (75%)	0	0
<i>Quercetin 5,7,3',4'-tetramethyl ether 3-rutinoside</i> <i>C31H38O16</i>	0	0	0	2 (50%)	0	0
<i>epicatechin 3-O-(4-O-methylgallate)</i> <i>C23H20O10</i>	0	0	0	0	12	12
<i>Methyl cinnamate</i> <i>C10H10O2</i>	0	0	0	0	12	9
<i>Catechin 3-O-alpha-L-rhamnoside</i> <i>C21H24O10</i>	0	0	0	0	1	0
<i>gibberellin A3 O-beta-D-glucoside</i> <i>C25H32O11</i>	0	0	0	0	0	0
<i>Cyanidin 3-(diferuloylsophorose) 5-glucoside</i> <i>C17H26N2O</i>	0	0	0	0	3	0
<i>3,4',6'-Trihydroxy-4,2'-dimethoxychalcone 4'-O-rutinoside</i> <i>C29H36O15</i>	0	0	0	0	4	0
<i>Campesteryl glucoside</i> <i>C34H58O6</i>	0	0	0	0	1	0

<i>Rhamnazin 3-rhamninoside</i> <i>C35H44O20</i>	0	0	0	0	1	0
<i>Cholesteryl glucoside</i> <i>C33H56O6</i>	0	0	0	0	1	0
<i>2,4-Dihydroxy-7,8-dimethoxy-2H-1,4-benzoxazin-3(4H)-one</i> <i>2-glucoside</i> <i>C16H21NO11</i>	0	0	0	0	1	0
<i>3-Methylpentyl glucosinolate</i> <i>C13H25NO9S2</i>	0	0	0	0	1	0
<i>Xanthoaphin</i> <i>C30H26O10</i>	5	0	0	0	2	0
<i>Acacetin 7-(2G-rhamnosyl)-rutinoside</i> <i>C34H42O18</i>	0	0	0	0	1	0
<i>5,7,3'-Trihydroxy-4'-methoxyflavanone 7-beta-L-rhamnosyl-</i> <i>(1->6)-glucoside</i> <i>C28H34O15</i>	0	0	0	0	1	0
<i>Epiafzelechin 3-O-beta-D-glucopyranoside</i> <i>C34H36F3NO13</i>	0	0	0	0	0	5
<i>Catechin-7-methyl ether</i> <i>C16H16O6</i>	5	0	0	0	0	8
<i>Phenethyl Rutinoside</i> <i>C20H30O10</i>	0	0	0	0	0	5

3,5,7,2',5'-Pentahydroxyflavone
C₁₅H₁₀O₇

0

0

0

0

0

7

6.5 Discussion

In this study, the dietary composition of SBY and its metabolic effects on a group of healthy participants was investigated. The test food (SBY) was developed by adding SBP to yogurt (a low GI food) and given to a group of 12 healthy participants (8 males and 4 females) in a cross-over design. Plain yogurt and white bread served as the control and the standard foods, respectively. To my knowledge, this is the first study to report the composition of and metabolic response to SBY.

It is well agreed that regular consumption of fruits is associated with a lower risk of many chronic diseases. This is especially true when inflammation and oxidation are major pathophysiological contributors. Type 2 diabetes mellitus, cardiovascular diseases, intestinal inflammation, as well as a wide range of cancers, are suspected to be positively impacted by fruit consumption, leading to a decreased incidence. The dietary antioxidants found in abundance in fruits are suspected of playing a major role in contributing to the protective/ curative roles of fruit consumption concerning oxidation and inflammation. According to the Canadian Diabetes Association's latest estimates, 22.1% of Canadians have pre-diabetes, and this trend is going upward (Canadian Diabetes Association, 2015). Diet is a key factor in the successful management of pre-diabetes, and the role of functional foods of low GI and high antioxidant capacity is no exception.

SBP is a product derived from one of the small fruits packed with dietary antioxidants, especially anthocyanins. One goal of the current study was to detect a group of phenolic compounds along with their metabolic products in both SBP and SBY. More than 24 phenolic compounds were found in all 5 SBP samples including the two key anthocyanins of SB. In SBY

alone, 22 phenolics found in SBP were retained even after long storage periods at -20°C. The retention of 22 phenolics is attributed to the acidity of SBY. Which was also good for another 15 phenolic compounds to exist and stand long-frozen storage periods, including flavonols, flavan-3-ols, anthocyanins, catechins and others detected in SBY, but not in the SBP. For a cultivated crop with a short harvest season and a high phenolic content, it is best to preserve the fresh produce in order to make it available all year round. Phenolic compounds of berries are better preserved if retained in acidic conditions. Yogurt is a low pH and low GI food and the addition of SBP to plain yogurt maintained a consistent pH value. It was reported that the pH of Saskatoon berry fresh fruits is 3.69 to 4.05 (St-Pierre, 2006). Freeze drying is considered one of the best preservation methods in terms of retaining the quality and the phenolic profiles of fruits (Michalczyk et al, 2009). A recent study found that freeze-dried SB retained the best content and activity of its antioxidants as demonstrated by retained DPPH and ABTS radicals scavenging activity when compared with other drying methods (Mitra et al, 2013). Proton NMR spectrometry was performed and the spectra confirmed the retention of phenolic compounds.

This is the first report on the phenolic content of SBY. The antioxidant capacity of SBY was tested to evaluate its potential as a functional food. Evaluating the antioxidant capacity of food may be a good tool to measure the additive antioxidant properties of fruits. It is recommended that two different methods be used to assess the antioxidant capacity of a food as this provides more reliable results and covers a wider range of molecules (oxidants) (Prior et al, 2005). DPPH radical inhibition (scavenging) capacity and the FRAP (ferric reducing antioxidant power) assays were utilized to assess the antioxidant capacity of SBY. DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical is one of the most stable nitrogen radicals. This method measures the ability of the food antioxidants (in this case, the small molecules) to reduce the DPPH radical.

DPPH radical inhibition is measured as a % based on the given equation (in the methods section). On a different note, the FRAP assay is used to evaluate the electron-donating ability of the antioxidants of food. The FRAP (ferric reducing/ antioxidant power) measures the ability of an antioxidant to reduce a complex of ferric iron and TPTZ to the ferrous iron (Moharram and Youssef, 2014). Findings of this study show that SBY had a significantly higher DPPH inhibition when compared to SBP. Furthermore, a sample of SBP is significantly more powerful as an antioxidant than if it was added to the yogurt. This indicates that the free radical scavenging capacity of the SBP antioxidants (most likely the small molecules phenolic compounds) is stronger than the SBY, and that adding the SBP to yogurt increased the functionality of the phenolic compounds. The effect of the phenolic compounds on the total antioxidant capacity was evaluated quantitatively. Three different amounts of SBP were tested alone or as SBY, assuming that a different amount of the SBP would have a different FRAP value, and the results confirm these assumptions. The FRAP value of 0.05 grams of SBP was higher than that of the SBY. In this case, the amount of SBP added to yogurt has an effect, and by testing 4.75 grams of the SBP it shows even higher antioxidant power. This may be attributed to the yogurt content of non-phenolic antioxidants that are better measured using the FRAP. In one report by Aloglu and Öner. (2011), yogurt exhibits an antioxidant activity attributed to its content of the antioxidant bioactive peptides, mainly contributed by the metabolic activity of lactic acid bacteria when used as a starter culture to ferment milk. The FRAP assay was used to test the antioxidant capacity of the species *A. canadensis* of the fresh Saskatoon berry fruits. The mean value was significantly higher compared to other berry fruits such as black mulberry and raspberry (Donno et al, 2016). When developing and testing a functional food, it is important to keep in mind the ability of the food vehicle to retain the active ingredients, phenolics in the current study, of the test food,

especially with the prolonged frozen storage periods from time of processing until time of ingestion, as this might destroy some of the phenolic content. Saskatoon berries were freeze-dried into a SBP, which was added to yogurt, and SBY was stored frozen for a few months.

The consumption of the berry fruits contributes to an enhanced intake of dietary antioxidant phenolic compounds. However, the metabolic effect of such compounds is not well investigated. Therefore, less is known about their post-ingestion occurrence in biological fluids such as plasma (Pimpão et al, 2015) and urine (Pimpão et al, 2014), and the mechanism(s) by which they modulate inflammation and oxidation (Carmen and Visioli, 2017). Of particular interest are the glycemic effect and the antioxidant response, pertinent to pre-diabetes. Flavonoids in berries (including anthocyanins) have been shown to regulate glucose absorption, and also to decrease glucose transporter gene expression *in vitro*. These mechanisms may lead to a modulated postprandial glycemic response after ingesting berries (Alzaid et al, 2013). In an *in vitro* experiment utilizing RAW264.7 cells, SB showed potent anti-inflammatory properties, which strongly inhibited TNF- α (Nayak et al, 2013). Two reports have been published using a diabetic mouse model (db/db diabetic dyslipidemic mice). The purpose of the latter studies was to test the protective and therapeutic potentials and their underlying mechanisms, of SBP. In one study, 5% SBP, when included in the regular diet, was able to attenuate the endoplasmic reticulum stress, induced by diabetes, as well as the unfolded protein response markers in the cardiovascular tissues of the diabetic mice (Zhao et al, 2015). In a parallel study, the results showed that the oral administration of SBP reduced monocyte adhesion, as well as inflammatory stress in cardiovascular tissues of diabetic mice (Zhao et al, 2014). Additionally, berries have been proven to lower plasma glucose, enhance antioxidant and anti-inflammatory activities,

decrease lipid oxidation biomarkers and improve insulin sensitivity (Hu et al, 2005; Dudonné et al, 2015).

Foods are categorized into three classes in terms of their GI: low ($GI \leq 55$), medium ($GI 56-69$), and high ($GI \geq 70$). GI was calculated according to Foster-Powell (Foster-Powell et al, 2002). The glycemic response is a measurement of the change in blood glucose levels following the consumption of digestible carbohydrates relative to a reference food with the same amount of available digestible carbohydrates. According to the findings of this study, SBY classifies as a low GI food, with a GI value of 36.4. This is the first report on the GI of SBY. In one serving, the total carbohydrate content is 25 grams, and this comprises 17 % of its total energy, coming from both simple sugars and complex carbohydrates of the freeze-dried SBP that is added to the yogurt. The glycemic load (GL) is the product of the GI of a food and its carbohydrate content. The GL is considered an indicator of the glucose response and the insulin demand in effect of 1 serving of the food (Barclay et al, 2008). Knowing that both SBY and plain yogurt fall within the same category (low GI), this suggests that the insulin demand might not be much different between the two foods, despite their different GL. Berry fruits fall in the low GI foods category (Törrönen et al, 2010). Despite their content of simple sugar (mainly fructose), their postprandial glycemic response is low. This is believed to be due to their phenolic content (Liderborg et al, 2012) which directly affects glucose absorption and metabolism (Liderborg et al, 2012; Törrönen et al, 2012). In this study, the SBY postprandial glycemic response, as well as the insulinemic response, was significantly lower after one hour of ingesting the SBY, compared to the standard (white bread), but not to the plain yogurt. Parallel studies further support similar findings, in a dietary interventional trial, a drink containing 47% bilberries (volume) resulted in a significantly lower insulin response, in comparison to white bread (Carmen and Visioli, 2017). The glycemic

response to a mixed berry purée versus sucrose drink as a control was tested in a group of healthy volunteers (n=12) (Törrönen et al, 2010). Plasma glucose concentrations were significantly lower after 15 and 30 minutes post-ingestion in the test group. This indicates that phenolics in the berry mix did contribute, in part, to the lower postprandial glucose response to 35 grams of sucrose in both foods. These data support a reduced absorption of sucrose from the berry purée (Törrönen et al, 2010). Plain yogurt, despite its content of available carbohydrates (25 grams), contained in 162.5 ml, was able to increase blood glucose levels more than SBY. This effect is most probably attributed to one or more ingredients found in the added SBP. In one trial, the inclusion of 40 g of dried lingonberries as part of a high glucose diet had a significantly lower insulin response (Linderborg et al, 2012). In another trial looking into the effect of strawberries and other berry fruit mixtures on plasma insulin, the test foods were shown to reduce insulin effectively (Yang and Kortensniemi, 2015). In this study, plasma insulin concentrations were higher than that of the SBY after 15 minutes of ingesting plain yogurt. This simply reflects the effect of sucrose that was added (1 tablespoon/ serving) to the plain yogurt to enhance the taste. A pitfall of this part of the analysis is the potential effect of the outlier on the final results. Two participants have reported they did not get enough sleep before one of their visits. Additionally, a couple more have reported they had to study for exams the night before their visit. Another limitation was the capillary blood collection. Typically, for the kit of choice (ELISA ultra-sensitive) used to measure insulin, a minimum blood collection of 60µl per sample (duplicate) is needed. In this case, three or more samples (multiple time points: 0, 15, 30, 60, 120, and 180 minutes) were below 60µl. This was discussed in detail previously (please see chapter 6). Altogether, the small sample size might have affected the accuracy and validity of these findings, especially for insulin concentrations.

The Canadian Diabetes Association recommends that people with diabetes increase their consumption of low GI foods (Bhattacharyya et al, 2008). It is becoming increasingly important the impact of the postprandial metabolic response of the berry diets on inflammatory markers, and the roles they play in inflammatory disease development. Owing to their low bioavailability, dietary anthocyanins' and polyphenols' roles as antioxidants *in vivo* have been controversial. Ingestion of foods and extracts rich with polyphenols has been associated with an increased plasma antioxidant capacity. However, in many reports scientists suggested that the bioavailability of dietary polyphenols is a concern, and it is normally too low to trigger significant endogenous antioxidant effect, especially given that the endogenous antioxidant system exceeds the millimolar range, while the dietary polyphenols were repeatedly reported to reach to the nanomolar range post absorption (Carmen and Visioli, 2017; Rodriguez-Mateos et al, 2014). In this study, there are no significant differences in plasma TAC and FRAP concentrations after ingesting SBY in comparison to the plain yogurt. In part, this can be due to the small sample size. In a parallel study examining the effect of blueberry juice consumption on plasma and urine antioxidant capacity using FRAP, a 500 ml serving of organic blueberry juice given to 9 healthy volunteers, did not show any effect compared to the sucrose solution (control), not after 30, 60, 120, and 240 minutes (Pedersen et al, 2000). Blueberry is the closest in composition to SB in terms of the polyphenol and nutrient profile. Despite giving a relatively high dose (500 ml) of the berry-containing food, FRAP concentration in the plasma of 9 volunteers (larger sample size) was still not statistically different. Dietary polyphenols are of low bioavailability and the measured circulating concentrations are normally in the nanomolar range (Tomás-Barberán and Andrés-Lacueva, 2012). The antioxidant/ anti-inflammatory effects exhibited by phenolic compounds of berries may be attributed to their local effect in the gut, as

the polyphenol gut microbiota metabolic products are better absorbed compared to their food polyphenols (Tomás-Barberán and Andrés-Lacueva, 2012). In one dietary interventional trial, 20 healthy volunteers showed a significant increase in their gut Bifidobacteria counts, as a result of regular consumption of a wild blueberry powder drink over a period of 6 weeks (Vendrame et al, 2011). In food, polyphenols occur mainly as esters, glycosides, and polymers, which are not normally absorbed in their forms. These parent compounds will need to be further digested by enzymes or intestinal microflora. An estimate shows that about 48% of the dietary polyphenols are hydrolyzed in the small intestine, while 42% are hydrolyzed in the large intestine (Tarko et al, 2013).

The utilization of LC-MS is regarded as one of the most suitable analytical approaches when the focus is to detect phytochemicals and their corresponding metabolites in foods, as well as in biological specimens. This approach allows the detection of a wide range of semi-polar compounds present in very low concentrations (Hanhineva, 2015). In this study, many of the anthocyanins, phenolic compounds products of digestion and hydrolysis were detected and appeared in plasma after 45 and 90 minutes in a subgroup of the study participants (n=4), including cyanidin and quercetin glucosides and their derivatives (table 6.6). Many of the dietary interventional trials on the absorption, circulation, metabolism, and excretion of dietary polyphenols focus on the metabolism in the small intestine and the liver. Typically, unchanged polyphenols would appear in plasma within a few hours as aglycones or conjugates (Duynhoven et al, 2010). After 3 hours of SBY ingestion, some phenolic compounds and many phenolic compounds products appeared in plasma and urine. Rutinose is a disaccharide that is normally conjugated with other anthocyanins (e. g. cyanidin and peonidin) in berries (Bakowska-Barczak and Kolodziejczyk, 2007). Rutinose was detected in SBY, and 180 minutes post ingestion in

plasma, as well as in urine. Rutinose occurrence in SBY but not in SBP indicates that the separation of the anthocyanin moiety took place in yogurt. In one participant out of 12, rutinose was detected 180 minutes after SBY ingestion, whereas 8 participants excreted the sugar within 3 hours of SBY consumption. This indicates that the sugar is absorbable, whether as such or as an anthocyanin conjugate. Epicatechin, while not detected as such in any of the food samples, was found in the plasma and urine of all the study participants 3 hours post-ingestion. Evidence from human and animal studies indicates the involvement of epicatechin (a flavan-3-ol flavonoid) in the regulation of blood glucose levels, and the improvement of insulin sensitivity (Cremonini et al, 2019). Overall, it is clear from human, animal, and cell model studies that berry anthocyanins, in addition to berry polyphenols other than anthocyanins, help regulate carbohydrate absorption and metabolism. When berries are consumed on a regular basis, findings from animal model studies and human studies indicated the involvement of anthocyanins, and their metabolites in the normalization of post-prandial glucose (Castro-Acosta et al, 2016).

6.6 Conclusion

SBY is introduced as a low GI food enriched with dietary phenolic compounds, and has lower glucose and insulin postprandial responses, compared to the bread with the same carbohydrate amount. The addition of the freeze-dried SBP is most likely responsible for the altered metabolic responses. Additionally, the antioxidant capacity of SBY is comparable to the SBP alone, and this indicates that the phenolic compounds are well retained in the yogurt, suggesting that yogurt is a good vehicle to deliver the phenolics of the SBP to consumers. The postprandial antioxidant response indicates that SBY might exhibit antioxidant and anti-inflammatory effects if tested in a larger sample for long term use. The occurrence of phenolics

and their relative metabolites in plasma after 45, 90, and 180 minutes, as well as after 180 minutes in urine, indicate that SBY might be metabolically functional, and it can be further investigated, developed and introduced as a low GI functional food. Overall, SBY may qualify for individuals with low-grade chronic inflammation, those who are on their way to develop diabetes or with an altered glucose response.

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Chapter 7: General Discussion

7.1 Overall discussion and conclusion

There is general consensus that the metabolic response to particular dietary components varies widely, even among individuals of the same age, sex and overall health status. Traditionally, the average response to a dietary intervention is usually predicted for a group of individuals, in many of the studies conducted on humans. However, the exact metabolic response(s) in each individual may vary dramatically, influenced by their genetic variations. Recent advances in research approaches exploring nutrigenetics/ nutrigenomics has led to the development of more tools to elucidate the nutrition-gene link in order to optimize health through the personalization of the diet. These tools provide potent explanations describing the complex link between genetic variations. Most common are the single nucleotide polymorphisms (SNPs), nutrients, and the whole biological system. This will increase the knowledge about the mechanisms leading to personalized diet management, or the so-called “Personalized Nutrition” (PN). A PN regiment seeks to tailor general nutrition recommendations to the exact biological needs of a person’s genetic makeup, phenotype, current health status, and future health outlooks. Therefore, when it comes to the current dietary recommendations, “One size fits all” is no more the case. PN is quickly becoming a reality for many reasons, including the recent advancement in technology (analytical research), scientific, and social developments that extend the current knowledge on public health promotion, dietary recommendations, and PN (Isabel et al, 2012; Verma et al, 2018).

In the current literature, few lines of evidence provide modelling approaches, where nutrient- metabolome- gene interaction has been successfully tested, and has led to the implementation of PN in the management of diseases associated with inflammation.

The main purpose of this research was to gain a better understanding of the link between dietary antioxidants and human health, and to reveal the relationship between antioxidants, metabolites, and genes. The recent advancements in research methodology, especially the “omics” technologies have provided a great set of molecular tools for nutrition research. The evolution of both “nutrigenomics” and “metabolomics” tools makes it possible to go far beyond basic nutrition research, and enables further advancements, including but not limited to: exploring molecular mechanisms and metabolic pathways, identifying novel biomarkers, the development and analysis of novel functional foods, and tracing primary as well as secondary metabolites following food consumption. The knowledge on the mechanisms by which diet or particular foods alter the various metabolic pathways is accumulating rapidly. This has led to the extensive knowledge being generated regarding the processes regulating the absorption and excretion of nutrients and their metabolites. Over the past few years, mass spectrometry-based metabolomics techniques have been used to a significant extent in both acute and chronic nutritional interventional studies. Plasma and urine analytical samples are the choice in most of the cases, with few studies analyzed fecal samples. Plasma analysis provides a wide picture of metabolic changes occurring in the circulation at a certain time point, particularly recent exposure, and reflects the response of the metabolome to a diet or food. The potential for these techniques to advance the development of functional foods is quite high.

In this research, 2 model studies have been utilized. In each, a novel analytical approach was utilized, looking at the knowledge gap from a different angle. In the first study, a “Nutrigenetics of ascorbic acid and IBD” approach served as the model of choice, and the topic of discussion in chapters 4 and 5. Ascorbic acid homeostasis is maintained in part by its transport through 2 of the SLC23 members: SLC23A1 and SLC23A2. However, the third family member

SLC23A3 and its coding gene *SLC23A3* have never been examined (orphan gene). According to gene databases (UniGene, AceView and ENSEMBL), tissue expression profiles of *SLC23A3* show a very similar pattern to those of its family member *SLC23A1*. Additionally, the shared DNA sequence homology amongst *SLC23A1* and *SLC23A3*, all together with the search for the basal membrane ascorbic acid transporter in the same tissues, formed the rationale for the search for the gene structure and association to IBD of *SLC23A3*. For the first time, the genomic structure of *SLC23A3* is described. *SLC23A3* is the smallest gene among its family members *SLC23A1* and *SLC23A2*, with a confirmed genomic size of 8.6 kilo bp. *SLC23A3* was found to be highly expressed in different segments of the epithelial tissues of the small intestine, large intestine, liver, and kidneys when measured in human biopsies. Three major SLC23A3 protein isoforms exist and vary in length and transmembrane domain structures: 618, 610 and 493 amino acids each.

Genetic association studies with a successful PN outcome were focused on people with metabolic disorders, including but not limited to: familial hypercholesterolemia, hypolactasia, and infant inborn errors of metabolism (phenylketonuria and maple syrup disease). These are examples of specific functional genetic disorders or genetic markers. More research directed towards identifying and testing the effect of the less common SNPs (biomarkers) is needed. This is particularly true for genes related to function regulation (homeostasis) of oxidative stress, inflammation, and metabolic pathways and new environmental factors affecting the SNPs already tested (San-Cristobal et al, 2013). Although a genetic variant in *SLC23A1* associated with CD, selected genetic variants of the *SLC23A3* did not associate with intestinal inflammation in Caucasians.

Unlike its family members SLC23A1 and SLC23A3, SLC23A2 is ubiquitously expressed in the body. It is responsible for ascorbic acid entry into cells, therefore contributing to an overall tissue concentration level as well as an antioxidant balance. Antioxidant balance is crucial in IBD, and a disturbed balance is a major contributing factor to disease onset and progression. Despite the wide tissue distribution of *SLC23A2*, as well as its association to several inflammatory conditions (Casabonne et al, 2017; Chen et al, 2019; Ravindran et al, 2018; Shaghghi et al, 2016), its genetic association to IBD has never been studied. In chapter 5, the variation of *SLC23A2* and IBD was discussed. Findings of this research revealed that 3'UTR variant rs35560557 (A-allele) associates with both CD and UC. The exact mechanism(s) by which variant rs35560557 contributes to IBD has yet to be evaluated. Further investigations of *SLC23A2* variation, regulation, and expression in IBD are very promising for its potential therapeutic effect(s).

These findings shed light on the possible mechanisms by which ascorbic acid personalized recommendation can become a reality in IBD prevention and management. Despite the availability of a number of research designs utilizing a personalized diagnosis and management approach, recommendations based on PN efforts are inconclusive, therefore not applicable. Practitioners, and especially dietitians, find it hard to make their recommendation based on robust evidence (Ommen et al, 2017; Verma et al, 2018). The diet, including both nutrients and non-nutrients, is integral to health, and dietary recommendations can be optimized at the individual level, taking into consideration a person's system and specific health outcomes (Ommen et al, 2017). In this regard, "Metabolic responses to Saskatoon berry yogurt" was the topic of discussion in chapter 6 and appendix C, and the second study model of choice.

SBY has been developed, analyzed and tested in a group of healthy participants. As a low GI food, SBY showed lower glycemic and insulinemic responses compared to the control. These health effects are mostly attributable to polyphenols. Polyphenols of SBY prove to be functional even after more than 6 months of frozen storage, indicated by the postprandial antioxidant response. SBY antioxidant response was assessed using two independent, robust techniques, TAC and FRAP antioxidant kits (for quantitative analysis), as well as the high throughput QTOF-MS (for qualitative analysis). Parallel clinical research findings are in line with the findings of this research. The consumption of a diet rich in berries and the associated antioxidants (anthocyanins) enhances insulin sensitivity, reduces plasma glucose and is inversely associated with diabetes mellitus type 2 (Kowalska et al, 2016). Evidence from *in vitro* and *in vivo* studies attributes these effects to the ability of berry extracts and their anthocyanin content to slow down glucose absorption at the intestinal lumen. Possible mechanisms imply that polyphenols interfere with glucose transporters, and that polyphenols also inhibit the activity of pancreatic amylases and glucosidases (Castro-Acosta et al, 2016). SBY is promising as a functional food not only for the healthy, but also it may qualify for persons with insulin resistance, glucose intolerance, and type 2 diabetes mellitus.

On a different note, anthocyanin-rich extracts of fruits, including berries, have been found helpful in the management of gastrointestinal inflammatory conditions including IBD. However, most of the evidence supporting the potential therapeutic efficacy of various berries in intestinal inflammation is based on animal models (Sodagari et al, 2015). As a final remark, despite a great deal of evidence supporting all these benefits of berry diets, there is still a boundary between nutrition recommendations and personalized management plans intended for health optimization, disease prevention and management in case of insulin resistance and

diabetes mellitus type 2. Improved research designs and outcomes hold the potential for addressing such a boundary.

7.2 Summary

Findings of this research have implications for developing personalized dietary recommendations, for IBD patients (first model), and for the healthy (second model).

The uniqueness of the findings of the study is that, it was the first to characterized a potential ascorbic acid transporter gene (*SLC23A3*). The current findings advance our understanding of the gene structure, splice variants, and tissue expression levels. This finding will prime for further investigating the function of the resulting protein (*SLC23A3*), and its potential substrate(s). Additionally, this is the first time variants of *SLC23A3* are tested for association with IBD.

Variant rs35560557 (A-allele) associates with both CD and UC. This SNP is located on 3' UTR region of the *SLC23A2*, and this is the first time *SLC23A2* is tested for association with IBD. The detection of genetic variations in persons with CD and/ or UC may indicate that the observation of low plasma and intestinal mucosal concentration of ascorbic acid may be more of a cause than an effect of IBD. Such a finding primes for further exploring the mechanism(s) by which *SLC23A2* contributes to IBD predisposition, disease onset, disease progression, and disease treatment through dietary intervention. Another, this may have implications for developing diagnostic tools (biomarkers) for early detection.

In the second model, a low GI functional food was developed and have been tested in human participants for the first time. SBY serves as an example of a novel functional food that has antioxidant properties, that can be further tested in persons with insulin resistance, prediabetes, and others. Our findings have implications on the development of low GI foods enriched with antioxidants.

7.3 Future direction

For the first study, a secondary analysis of data sets that were collected several years ago was performed. The original study “The Manitoba IBD Cohort Study” was initiated in 2005, and DNA samples were collected from almost all eligible study participants. In the first research project, we have analyzed 822 samples at a ratio of 1:1:1 as CD cases: UC cases: matching controls. The original question to be answered was whether an association exists between SNPs of the *SLC23A2* and *SLC23A3* and IBD. Findings indicated the existence of an association of the *SLC23A2* variant rs35560557 (A-allele) in a group of Caucasian people diagnosed with IBD. Looking at the sample size, although it allows for precision and accuracy in answering the research question, it is not the ideal sample size compared to other genome-wide association studies.

For the future, researching various population groups will better answer the question. Additional questions arise here: 1) How is this SNP rs35560557 putting people at risk for UC or CD? 2) Is it affecting plasma ascorbate levels directly? 3) Is it affecting ascorbate uptake in the intestines? 4) Is it affecting the colonic bacteria and host defenses?

Collecting plasma samples meant for ascorbic acid measurement, in addition to a dietary intake assessment of vitamin C and other antioxidant nutrients, and an overall nutritional status is sought. Additionally, *in vitro*, *in vivo*, and *ex vivo* experiments will help identify the exact role(s) of variant rs35560557 in *SLC23A2* expression patterns and levels in various tissues, and will help reveal protein functionality, alternative splicing or other potential mechanisms affecting *SLC23A2*. Eventually, this will help better understand the impact this might have on the inflammatory events of the intestines.

The second study was designed to test the functionality of the newly developed SBY in a

group of healthy volunteers for the first time. As a pilot study, the sample size of 12 healthy participants is considered ideal, but not when insulin is a parameter. Insulin circulating levels can be easily affected by several factors such as having an inappropriate sleep the night before the study visit, or being stressed out emotionally or physically. For the future, a more extensive study sample size (minimum of 24 participants), and better blood collection (typically venous blood collection) would provide more valid results.

For antioxidant response, a sub-sample of 4 participants was used for this part of the study. Quantitatively, the amount of SBP used was approximately 68 grams in a cup (1 serving) given to each of the participants. Sixty-eight grams of freeze-dried SBP result from the almost 120 grams of the fresh fruit, this converts into $\frac{3}{4}$ of a cup of the fresh berries. Over 50 of the polyphenolic constituents were successfully detected in the participants' blood and urine in effect of SBY through QTOF-MS, versus none in effect of yogurt. However, when we look at the plasma antioxidant response, it is not showing any statistical difference. On the other hand, most of the phenolic compounds will reach their peak plasma levels within 3-6 hours post-ingestion (Castro-Acosta et al, 2016). In this study, the last measurement was taken 180 minutes post-ingestion (3 hours). Many of these compounds or their metabolites might have been missed. For the future, considering a larger sample size, along with detecting the occurrence of polyphenols in plasma after 3-6 hours, would result in better findings.

Drawing on the findings from both of the study models, the optimal development of PN recommendations requires more information on the bioavailability of dietary antioxidants. In this case, ascorbic acid and bioactives of SB in people with intestinal inflammation, and in healthy participants, respectively. To achieve this, more information about nutrient intake and its functional and physiological effects is needed, and an integral part of which is the search for

valid biomarkers (relevant SNPs and primary or secondary metabolites). Despite the successful use of the currently existing “traditional biomarkers”, such as serum zinc and folate, not all individuals in a physiological group would have the same effects.

The establishment of a comprehensive tool to be utilized for the nutritional status assessment, leading to a successful PN for metabolic disease management is not easy. Challenged by the limited knowledge available on the influence of genes on nutrient utilization, and how this would reflect upon the metabolic profile as well.

The generation of more data, driven by nutrigenomics, metabolomics, proteomics, and transcriptomics research, may enable the practitioners to use PN in disease management rationally. This will be especially true if applied to larger populations in well-designed interventional trials. With more knowledge accumulating, the use of the findings will also help reduce the risk factors contributing to metabolic disorders. However, and despite the limitations, the potential of utilizing “omics” approaches to develop a comprehensive PN platform for disease prevention and treatment is still very promising.

7.4 References

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APPENDICIES

APPENDIX A: Ethical approval forms



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HEALTH RESEARCH ETHICS BOARD (HREB) CERTIFICATE OF ANNUAL APPROVAL

PRINCIPAL INVESTIGATOR: Dr. Charles Bernstein	INSTITUTION/DEPARTMENT: U of M and HSC/Internal Medicine	ETHICS #: HS11759 (H2008:165)
HREB MEETING DATE (if applicable):	APPROVAL DATE: June 11, 2018	EXPIRY DATE: June 23, 2019
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (if applicable):		

PROTOCOL NUMBER: Na	PROJECT OR PROTOCOL TITLE: The Manitoba IBD Cohort Study and The Manitoba Newly Diagnosed IBD Study (Inception Cohort) (Linked to H2002:040)
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: CIHR	

Submission Date of Investigator Documents: May 9, 2018	HREB Receipt Date of Documents: May 15, 2018
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REVIEW CATEGORY OF ANNUAL REVIEW: Full Board Review ☐ Delegated Review ☒

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

Document Name(if applicable)	Version(if applicable)	Date
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Annual approval

*Annual approval implies that the most recent **HREB approved** versions of the protocol, Investigator Brochures, advertisements, letters of initial contact or questionnaires, and recruitment methods, etc. are approved.*

Consent and Assent Form(s):

CERTIFICATION

The University of Manitoba (UM) Health Research Board (HREB) has reviewed the annual study status report for the research study/project named on this **Certificate of Annual Approval** as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. Annual approval was granted by the Chair or Acting Chair, UM HREB, per the response to the conditions of approval outlined during the initial review (full board or delegated) of the annual study status report.

HREB ATTESTATION

The University of Manitoba (UM) Health Research Board (HREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the HREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

Research Ethics and Compliance is a unit of the Office of the Vice-President (Research and International)

umanitoba.ca/research

QUALITY ASSURANCE

The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. ***For logistics of performing the study, approval must be sought from the relevant institution(s).***
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. **This approval is valid until the expiry date noted on this certificate of annual approval.** A Bannatyne Campus Annual Study Status Report must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the HREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form**.
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM HREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report**.

Sincerely,

Bannatyne Campus



UNIVERSITY
OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Board

P126 - 770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Telephone 204-789-3255
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BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF FINAL APPROVAL FOR NEW STUDIES
Full Board Review

PRINCIPAL INVESTIGATOR: Dr. Carla Taylor	INSTITUTION/DEPARTMENT: U of M and SBRC/Department of Nutritional Sciences	ETHICS #: B2014:121
BREB MEETING DATE: November 24, 2014	APPROVAL DATE: January 21, 2015	EXPIRY DATE: <u>November 24, 2015</u>
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable):		

PROTOCOL NUMBER: SBFY-2014	PROJECT OR PROTOCOL TITLE: A Randomized, Controlled Study to Examine the Glycemic and Metabolic Responses of Frozen Yogurt Containing Saskatoon Berry Powder in Healthy Males
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Manitoba Agri-Health Research Network (MAHRN)	

Submission Date(s) of Investigator Documents: October 31, 2014 and January 6, 2015	REB Receipt Date(s) of Documents: November 3, 2014 and January 7, 2015
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THE FOLLOWING ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol: Protocol	1.1	January 6, 2015
Consent and Assent Form(s): Research Subject Information and Consent Form	1.1	January 6, 2015
Research Subject Information and Consent Form for Collection, Storage and Use of Biological Materials for Metabolomics Research	1.1	January 6, 2015
Other: Website Advertisement	1.1	January 6, 2015
Poster Advertisement	1.0	October 27, 2014
WRHA CORPCOM E-Advertisement	1.0	October 27, 2014
Initial E-mail /Mail response script	1.0	October 27, 2014
Questionnaire	1.0	October 27, 2014
3-day Food Record	1.0	October 27, 2014

CERTIFICATION

The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the research study/project named on this **Certificate of Final Approval** at the **full board meeting** date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM BREB.

BREB ATTESTATION

The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba.

In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE

The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. ***For logistics of performing the study, approval must be sought from the relevant institution(s).***
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. **This approval is valid until the expiry date noted on this certificate of approval. A Bannatyne Campus Annual Study Status Report** must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form**.
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report**.

Sincerely,



Chair, Biomedical Research Ethics Board
Bannatyne Campus

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Please quote the above Human Ethics Number on all correspondence.

Inquiries should be directed to the REB Secretary Telephone: (204) 789-3255/ Fax: (204) 789-3414

APPENDIX B: Gene analysis software tools

<i>Tool</i>	<i>Link</i>	<i>Application</i>
<i>Biobase®</i> <i>TRANSFAC</i>	http://www.biobase-international.com/	Promoter region prediction (gene)
<i>Center for biological sequence bioinformatics tools- technical university of Denmark</i>	http://www.cbs.dtu.dk/	Regulatory elements prediction (gene)
<i>BLAST®</i>	https://blast.ncbi.nlm.nih.gov/	Amino acid alignment and comparison (protein)
<i>DAS transmembrane server</i>	http://www.sbc.su.se/	Transmembrane domain prediction (protein)
<i>Protter</i>	http://wlab.ethz.ch/	
<i>Octopus</i>	http://octopus.cbr.su.se/	
<i>Sequencher® 5.1</i>	http://www.genecodes.com/	DNA sequence analysis

APPENDIX C: NMR based quantitative investigation of urine metabolites in effect of SBY

C.1 Introduction

Metabolomics is the study of systems metabolites (chemical interactions) in the body (Austdal et al, 2014). NMR spectrometry is one of the novel metabolomics analytical techniques. NMR is used in human studies, and is very promising as a tool for identifying and quantifying metabolic profiles. Findings from dietary interventional trials have been able to identify a number of health-promoting compounds: nutrients and non-nutrients in the diet. However, in most of the studies the total effect of the treatment diet could not be attributed by a single compound, or even a group of compounds (Paudel et al, 2013). The use of NMR spectrometry holds potential for the detailed phenotyping of the metabolic profiles in effect of a test food (SBY in this example), or a diet. It makes the search for the whole set of metabolites that can promote health or help manage disease more feasible (Lehtonen et al, 2013; Paudel et al, 2013). From this investigation, the aim was to quantify a group of metabolites in effect of plain yogurt versus SBY, in a group of healthy participants.

C.2 Methods

C.2.1 Study design and protocol

A single-site, randomized, controlled interventional post-prandial design, with 4 intervention visits and 1-2 screening visits was conducted during 2015 and 2016. The study protocol was approved by the Research Ethics Board (REB) at the University of Manitoba, and the St. Boniface Hospital Research Review Committee. The study was registered on ClinicalTrials.gov (<https://clinicaltrials.gov>), and given the code (NCT02571114).

C.2.2 Participants

12 healthy participants (8 males and 4 females) were recruited at the St. Boniface hospital (I. H. Asper Clinical Research Institute) through local advertisement. Recruitment started in March (2015) and ended by June (2016). The inclusion criteria were: healthy individuals aged 18-35 years, non-smoking, with no history of diabetes, metabolic syndrome or any other chronic metabolic diseases. Male and female (not pregnant or lactating) volunteers were screened for being generally healthy, assessed by a screening questionnaire, body mass index (normal to overweight), blood pressure, standard hematology, and liver and kidney functions. The participants have been on stable regimen for any nutritional and herbal supplements over the past 3 months prior to the study period (almost 6 weeks). All participants (n=12) signed a written consent form and completed the study, which included not consuming Saskatoon berries or any of its products while participating in the study. The participant demographics are shown in table C (1).

Table C (1): Baseline characteristics of study participants

<i>Characteristics (n=12)</i>	<i>Average</i>	<i>Minimum</i>	<i>Maximum</i>
<i>Males (n=8)</i>			
<i>Females (n=4)</i>			
<i>Age (years) Males</i>	27.9	21.0	34.0
<i>Age (years) Females</i>	25.3	20.0	32.0
<i>Weight (kg) Males</i>	79.6	58.0	99.0
<i>Weight (kg) Females</i>	64.1	59.5	69.0
<i>BMI^a (Kg/m²) Males</i>	25.41	20.3	29.7
<i>BMI (kg/m²) Females</i>	25.2	23.5	28.7
<i>Blood pressure (mmHg)^b Males</i>	113/78	97/67	127/86
<i>Blood pressure (mmHg) Females</i>	105/70	91/57	111/81
<i>HbA1c^c Males</i>	5.2	4.6	5.5
<i>HbA1c Females</i>	5.3	5.2	5.3

^a BMI: Body mass index was calculated using weight in kilograms divided by height in meters squared.

^b mmHg: Millimeters mercury.

^c HbA1c: Glycated hemoglobin A1c.

C.2.3 Urine sample collection and extraction

Urine samples were collected using toilet hat specimen collectors before food consumption and 3 hours after the participants had consumed the test food (n=12). Urine was separated into smaller aliquots of 2 and 5 ml, labelled and kept in the -80 °C until being used for further analyses. For extraction analysis, urine samples were thawed on ice, 250µl aliquots (duplicates) were vortexed with 10µl D-norvaline (standard) for 10 seconds, then samples were centrifuged for 10 minutes at 10000xg. Supernatants were mixed with 500µl of acetonitrile and

vortexed for 10 seconds and kept in the -20 °C for 30 minutes for quenching, after that samples were centrifuged at 10000xg for 20 minutes (room temperature), dried under nitrogen flow and reconstituted with 200µl of (4:1, v/v) water: acetonitrile.

C.2.4 NMR analysis

400µl of urine sample (kept in the – 20 °C, thawed on ice) was added to 230µl 0.2M phosphate buffer of D2O and 70µl of Chenomx® ISTD solution. The mixture was vortexed for 30 seconds, centrifuged at 1200 rpm for minutes. 600µl of sample was transferred into 5mm NMR tubes (Bruker). Tubes were covered and inserted into the ¹H NMR device, ¹H NMR spectra were recorded with a Bruker™ ASCEND 600 MHz NMR magnet system operating at a proton frequency of 600.13 MHz. for statistical analysis, metabolite concentrations were analyzed in SPSS (version 23 for Mac), using one-way ANOVA. Urine metabolite concentrations used were adjusted to creatinine as [metabolite/ creatinine] to correct the dilution/concentration. P<0.05 was considered significant.

C.3 Results

Characteristics of the study participants were given previously (table C (1)). Urinary metabolite concentrations of 9 metabolites (including creatinine) are shown in table C (2). Baseline metabolite concentrations were not significantly different prior the consumption of yogurt and the test food (SBY).

Table C (2): Urine metabolite concentrations in effect of yogurt and SBY (n=12)

<i>Metabolite (μM/mM creatinine)</i>	<i>Yogurt ± SEM</i>	<i>SBY ± SEM</i>	<i>P value</i>
<i>Alanine</i>	0.03 ± 0.01	0.60 ± 0.20	<0.05
<i>Betaine</i>	0.02 ± 0.01	0.18 ± 0.10	<0.05
<i>Citrate</i>	0.15 ± 0.04	1.74 ± 0.50	<0.05
<i>Hippurate</i>	0.07 ± 0.02	1.41 ± 0.41	<0.05
<i>Glycine</i>	0.11 ± 0.03	1.83 ± 0.53	<0.05
<i>Lactate</i>	0.02 ± 0.01	0.26 ± 0.08	<0.05
<i>Methylmalonate</i>	0.20 ± 0.01	0.12 ± 0.03	<0.05
<i>Taurine</i>	0.08 ± 0.02	11.35 ± 3.30	<0.05
<i>Uracil</i>	6.70 ± 1.94	1.12 ± 0.32	<0.05
<i>Creatinine</i> [*]	6.73 ± 1.94	2.00 ± 0.60	<0.05

Values represent mean concentration ± SD.

*Creatinine: absolute value (not adjusted for dilution)

C.4 References

- Austdal, M., Skrastad, R., Gundersen, A. et al (2014). Metabolic biomarkers in serum and urine in women with preeclampsia. *PLOS ONE Journal*, 9(3), 1-7.
- Lehtone, H., Lindstedt, A., Jarvinen, R. et al (2013). ¹H NMR-based metabolic fingerprinting of urine metabolites after consumption of lingonberries (*Vaccinium vitis-idaea*) with a high-fat meal. *Food Chemistry*, 138, 982-990.
- Paudel, L., Wyzgoski, F., Giusti, M. et al (2013). NMR-based metabolomics investigation of bioactivity of chemical constituents in black raspberry (*Rubus occidentalis*) fruit extracts. *Agricultural and Food Chemistry*, 62, 1989-1998.*