Genetic and functional studies of two intestinal vitamin C transporters, SLC23A1 and GLUT14, associated with inflammatory bowel disease

By

Mandana Amir Shaghaghi

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg

ABSTRACT

It had long been known that individuals with inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), have locally reduced vitamin C levels in the intestinal mucosa, which equates to an overall loss of the antioxidant capacity and increase risk of oxidative tissue damage. The aim of the present work was to expand on this concept, through investigating the role of genetic variations in intestinal vitamin C transporters in vulnerability to IBD and further characterizing their functions. The studies focused on a known intestinal transporter for ascorbic acid, *SLC23A1*, and a novel intestinal transporter of dehydroascorbic acid, *SLC2A14* (GLUT14). To investigate any association between the *SLC23A1* and *SLC2A14* with IBD, genomic DNA of 311 Caucasian individuals with IBD, participated in the Manitoba IBD Cohort Study, and 142 healthy controls were genotyped for tagging single nucleotide polymorphism (SNP) of each gene, using TaqMan Assays. New splice variants of *SLC23A1* and *SLC2A14* were determined by *In silico* analyses, followed by sub-cloning of the splice variants to verify their subcellular locations. Substrates and functions were determined, using the Xenopus laevis oocyte system for each transporter. The presence of the *SLC23A1* variant rs10063949 G allele elevated the risk for CD by 150% (Odds ratios (OR) = 2.54, 95% CI 1.83-3.53). An allele dosage effect was confirmed; compared to rs10063949-AA homozygotes the 10063949-AG heterozygotes have a 150% elevated risk and the 10063949-GG homozygotes have a 370% elevated CD risk (OR=2.54, 95% CI 1.38-4.66; OR=4.72, 95% CI 2.53-8.81, $p<0.001$; respectively). No relation was observed between genetic variants in *SLC23A1* and UC. Through database search, a novel 5'exon was discovered for *SLC23A1* locus which is located 1078 nucleotides upstream of the canonical first exon. The two first exons are not mutually

exclusive since they splice together to create a novel SLC23A1 protein isoform, we named it isoform 1A, with a N-terminus that is elongated by 36 amino acid. The novel SLC23A1 isoform located at the plasma membrane, but mediates only 7% of the ascorbic acid transport exhibited by the shorter isoform. The presence of the *SLC2A14* SNP rs2889504 A allele elevated the risk for UC by 260% and CD by 468% (OR: 3.60, 95% CI: 1.95-6.64; OR: 4.68, 95% CI: 2.78-8.50, respectively). The rs10846086-G allele elevated the risk of UC and CD approximately 3-fold (OR: 2.91, 95% CI: 1.49-5.68; OR: 3.00, 95% CI: 1.55-5.78, respectively). The variant rs12815313-T increased the risk for CD by 112% (OR: 2.12, 95% CI: 1.33-3.36). All the genetic variations in *SLC2A14* gene, associated with IBD, were independent from each other, strengthening the evidence that functional SNPs in the *SLC2A14* locus contribute to IBD. It was identified that the two major GLUT14 isoforms locate to the plasmalemma membrane and mediate cellular uptake of dehydroascorbic acid. Significant expression in extra-testicular tissues was confirmed for *SLC2A14*, notably in intestinal segments, explaining the association with IBD. Re-analysis of genomic showed a dramatically expanded locus of *SLC2A14*, containing twenty exons which covered 103,477 nucleotides from the first Transcriptional Start Site (TSS) to the termination of the longest transcript. All together, the presented evidence indicate that functional SNPs in the *SLC2A14* gene and *SLC23A1* could contribute to vitamin C imbalance in mucosal cells which contributes to an elevated risks of IBD. Furthermore, novel information about genetic and functional characteristics of SLC23A1 and GLUT14 transporters was identified.

ACKNOWLEDGMENTS

There are several people to whom I would like to express my appreciation for their support and inspiration throughout my PhD program. I am sincerely thankful to my supervisor, Dr. Peter Eck, for providing me excellent supervision, outstanding support, exemplary encouragement and exceptional opportunities throughout the duration of my doctoral studies at the University of Manitoba. Peter, you have taught me how to think outside the box, look at any research gap from many different angles, and love the research with my whole heart. It has been an incredible journey and I truly thank you! I would also like to extend a big thank you to my co-supervisor, Dr. Peter Jones, who has remarkably supervised/co-supervised me during my whole adventure as graduate student at University of Manitoba. Dr. Jones, you have always guided me to passionately work harder, be stronger and not being afraid of any challenge. My pathway toward being a researcher doesn't have any meaning without you! My deep appreciation goes to my committee members, Dr. Semone Myrie, for your precious mentorship and inspirational support, and Dr. Ehsan Khafipour, for your valuable guidance and insightful advice. I would like to sincerely thank my external examiner, Dr. Marie-Claude Vohl at Université Laval, for agreeing to review and evaluate this thesis and to accommodate the defense date in your tight schedule. I would also like to acknowledge Dr. James House, Dr. Charles Bernstein, and Dr. Garry Crow for their excellent guidance. I am also grateful to the staff and faculty of the Department of Human Nutritional Sciences at the University of Manitoba, specifically Mrs. Pam Gauthier and Mrs. Janelle Ritchot. Above all, this could not have been possible without the financial support and scholarship from NSERC/FAST training as well as several scholarships from Faculty of Graduate studies, Faculty of Human Ecology, and American Society for Nutrition.

DEDICATIONS

I dedicate this work to my family whom I have been blessed beyond measure

by their presence in my life.

To my husband and best friend Shahrokh.

To my mother Minoo and my late father Razi.

To my brother Reza.

I would not have been able to do this without your continuous love and undying support.

FOREWORD

This thesis is written in manuscript style and is composed of eight chapters and five manuscripts. The first chapter includes "Overall Introduction" followed by chapter two "Literature Review" which will critically assess the existing body of evidence surrounding the inflammatory bowel disease and vitamin C. Thereafter, the manuscripts, addressing specific objectives, will be presented. A one page transition statement at the end of each manuscript links the following chapter for a consistent flow. The last chapter encloses an "Overall Conclusion" of the thesis with concluding remarks of the work, its limitations, and proposed future directions of the research project.

List of Authors of each manuscript

- Manuscripts 1 (in press: Journal of Advances in Nutrition)

  Mandana Amir Shaghaghi[a], Olena Kloss[a], Peter Eck[a]

- Manuscript 2 (published: Journal of Human Nutrition & Food Science; J Hum Nutr Food Sci 1: 1001)

  Mandana Amir Shaghaghi[a], Hongbin Tu[b], Natalia Yurkova[a], Mark Levine[b], Peter Eck[a]

- Manuscript 3 (published: American Journal of Clinical Nutrition; Am J Clin Nutr 2014;99:378–83)

  Mandana Amir Shaghaghi [a], Charles N. Bernstein[c], Alejandra Serrano León[a], Hani El-Gabalawy [d], Peter Eck[a]

- Manuscript 4 (under revision: Biochemistry and Cell Biology)

  Mandana Amir Shaghaghi [a], Brent Murphy [e], Peter Eck [a]

- Manuscript 5 (submitted: American Journal of Human Genetics)

  Mandana Amir Shaghaghi [a], Hongbin Tu[b], Charles N. Bernstein[c], Hani El-Gabalawy[d], Garry Crow[f], Mark Levine[b], Peter Eck[a]

Afflications: [a]Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, R3T 2N2. [b]Molecular and Clinical Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA. [c]University of Manitoba, IBD Clinical and Research Centre.[d]Department of Internal Medicine, University of Manitoba, Winnipeg, Canada, R3E 3P4. [e]Department of Plant Science, University of Manitoba, Winnipeg, Canada.[f] Department of Animal Sciences, University of Manitoba, Winnipeg, Canada.

Corresponding author for all the manuscripts: Peter Eck, Department of Human Nutritional Sciences, W569 Duff Roblin Building, 190 Dysart Road, University of Manitoba, Winnipeg, MB R3T 2N2 Canada. E-mail: peter.eck@ad.umanitoba.ca Phone: +1-204-474-8079

List of financial supports for all the manuscripts: Canada Research Chair, Peter Eck; Food Advancement through Science and Training (NSERC-CREATE- PhD fellowship), Mandana Amir Shaghaghi

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AA	Acceptor Splice Site
AD	Alternative Donor Splice Site
ASC	Ascorbic Acid (Ascorbate)
BLAST	Basic Local Alignment Search Tool
CD	Crohn's Disease
CHO-K1	Chinese Hamsters Ovary Cells
cDNA	Complementary Deoxyribonucleic Acid
cRNA	Complementary Ribonucleic Acid
DHA	Dehydrascorbic Acid
ES	Exon Skipped
EST	Expressed Sequence Tags
FBS	Fetal Bovine Serum
GLUTs	Facilitative Glucose Transporter Family
GSH	Glutathione S-transferases
HEK	Human Embryonal Kidney Cells
HIF-1A	Hypoxia-Inducible Factor 1α
HPV16	Human Papillomavirus Type 16 Infection
IBD	Inflammatory Bowel Disease
IR	Intron Retention
KDa	Kilo Dalton
LD	Linkage Disequilibrium

MA	Mutually Exclusive Exons
NADH-	Nicotinamide Adenine Dinucleotide
NADPH-	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	The National Center for Biotechnology Information
OR	Odds Ratios
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCARB1	The Gene Scavenger Receptor Class B
sFPKM	significant Fragments Per Kilobase of Exon Per Million Fragments Mapped
SLC23A1	Solute Carrier 23 A1 (Sodium Dependent Ascorbic Acid Transporter)
SNP	Single Nucleotide Polymorphism
TSS	Transcriptional Start Site
TGFP	Turbo Green Fluorescent Protein
UC	Ulcerative Colitis
VISTA	Visualizing Global DNA Sequence Alignments Of Arbitrary Length

CHAPTER 1

OVERALL INTRODUCTION

1.1 INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic immune pathologies of the gastrointestinal tract commonly thought to depend on the interaction of environmental, genetic and immunological factors (1). Yet, an integrated concept explaining the etiology of IBD in relation to the pathophysiological changes has not emerged (2). It does seem certain that IBD is amplified and propagated by an uncontrolled host immune response, resulting in an extensive inflammatory infiltrate in the lamina propria, including polymorphonuclear neutrophils, eosinophils and plasma cells (3). Furthermore, oxidative stress through an excessive release of reactive oxygen metabolites is responsible for leading such an extreme and enduring mucosal immune activation to the excessive cellular and tissue damage, destruction of normal tissue and chronic inflammation that is observed in IBD (3-5). In individuals with IBD, a decrease in vitamin C concentrations in the intestinal mucosa (6) and in the plasma (7) has been reported, contributing to the overall loss of its antioxidant capacity. Daily supplementation of vitamin C resulted in significant decrease in oxidative stress (8) in individuals with IBD.

Vitamin C is an essential micronutrient and a critical cofactor for several intracellular enzymatic reactions. Ascorbic acid (ASC, reduced form) and dehydroascorbic acid (DHA, oxidized vitamin C) are the two dietary sources of vitamin C in humans. Both forms of vitamin C are absorbed from the intestinal lumen and renal tubules through enterocytes and renal epithelial cells, respectively (9, 10). Ingestion of ASC or DHA raises the plasma concentration of ASC (the predominant form of vitamin C at physiological pH) which then enters and accumulates in

almost all the body cells to function as an enzyme cofactor and antioxidant (11, 12). More important are specific mechanisms of transport system, associated with cells' plasma membranes, which determine the distribution of ASC between extracellular and intracellular fluids. Active transport of ASC and facilitated diffusion of DHA are the known transport mechanisms mediated by sodium dependent ascorbic acid transporters and facilitative glucose transporters family (GLUTs), respectively, which contribute to the membrane transport of vitamin C. Human enterocytes contain reductases that convert DHA to ASC (6, 13). This conversion mechanism boosts intracellular ASC dramatically and augments resistance to oxidative stress while maintaining the intracellular level of DHA low resulting in a concentration gradient which favors uptake of more DHA across the enterocytes' plasma membrane (14). Intestinal DHA absorption may be especially important during inflammation in IBD patients where chronic inflammation and excessive oxidative stress may accelerate oxidation of ASC to DHA resulting in reduced ASC concentration in inflamed mucosa of individuals with IBD (6).

The active transport of ASC across cell membrane is mediated by two sodium-dependent ascorbate transporters, SLC23A1 and SLC23A2, transporting ASC against a concentration gradients into cells (15, 16). SLC23A1 is the main intestinal ascorbic acid transporter on the apical side of the enterocyte, and some of the genetic variants of the *SLC23A1* gene have been associated with decreased ASC transport and lowered systemic levels (17-20). DHA, on the other hand, is transport through some member of GLUT family including GLUT 3 (21). Orphan GLUT14, encoded by *SLC2A14* gene, is the most recent known member of the GLUTs. GLUT 14 is human specific and has a very similar gene structure to GLUT 3 (95.5%) (22). Beside basic information on gene structure, basic biology and the substrate of GLUT 14 need to be

determined, however, based on the homology to GLUT 3, it looks reasonable to hypothesize that GLUT 14 is also a DHA transporter.

In this research project, we speculated that variation in vitamin C transporter genes would alter the transporter activity and result in localized intestinal antioxidants imbalance which will then contribute to the etiology of IBD.

1.2 RATIONALE

Nonetheless, the role of variants in vitamin C transporter genes on the mucosal antioxidant cascade respond to oxidative stress in individuals with IBD is a neglected area of research. This particular aspect of intestinal inflammatory conditions therefore merits additional study, in order to grant a sound, scientific basis for the design of vitamin C directed nutritional intervention strategies for individuals with IBD. To support the hypothesis that variations in vitamin C transporters may downregulate each transporter activity, results in redox imbalance, and thus increased susceptibility to IBD, we investigated the association between variations in two different vitamin C transporter (sodium dependent ASC transporter *SLC23A1* gene, and facilitative diffusion DHA transporter *SLC2A14* gene) with IBD risk, through genotyping the participants in a Caucasian cohort study. Also, to validate any possible association, a complete annotation of the each gene locus and function was performed.

1.3 OBJECTIVES

The present research has 4 specific objectives:

1. Validate alternative splice variants of *SLC23A1* gene.

2. Investigate the association of *SLC23A1* gene with susceptibility to IBD.

3. Examine the basic biology, function, and substrate of orphan GLUT14 transporter.

4. Determine the association of variants in the *SLC2A14* gene with IBD.

1.4 HYPOTHESES

The hypotheses to be tested include:

1. There are undetermined alternative splice variants for *SLC23A1* transcript which may have different transport activity and thus would impact intracellular vitamin C levels.

2. Genetic variations in the ascorbate transporter *SLC23A1* modulate transport activity and thus are associated with IBD risk.

3. *SLC2A14*, encoding GLUT14, has a defined gene locus and is expressed in tissues other than testis.

4. GLUT14 is a membrane protein mediating dehydroascorbate uptake.

5. Genetic variations in the dehydroascorbate transporter SLC2A14 alter transport activity and are associated with IBD.

1.5 REFERENCES

1. Fiocchi, C. 1998. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 115:182-205.

2. Sartor, R.B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 3:390-407.

3. Kruidenier, L., and Verspaget, H.W. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 16:1997-2015.

4. Jena, G., Trivedi, P.P., and Sandala, B. 2012. Oxidative stress in ulcerative colitis: an old concept but a new concern. *Free Radic Res* 46:1339-1345.

5. McCord, J.M. 2000. The evolution of free radicals and oxidative stress. *Am J Med* 108:652-659.

6. Buffinton, G.D., and Doe, W.F. 1995. Altered ascorbic acid status in the mucosa from inflammatory bowel disease patients. *Free Radic Res* 22:131-143.

7. Wendland, B.E., Aghdassi, E., Tam, C., Carrrier, J., Steinhart, A.H., Wolman, S.L., Baron, D., and Allard, J.P. 2001. Lipid peroxidation and plasma antioxidant micronutrients in Crohn disease. *Am J Clin Nutr* 74:259-264.

8. Aghdassi, E., Wendland, B.E., Steinhart, A.H., Wolman, S.L., Jeejeebhoy, K., and Allard, J.P. 2003. Antioxidant vitamin supplementation in Crohn's disease decreases oxidative stress. a randomized controlled trial. *Am J Gastroenterol* 98:348-353.

9. Wilson, J.X. 2005. Regulation of vitamin C transport. *Annu Rev Nutr* 25:105-125.

10. Malo, C., and Wilson, J.X. 2000. Glucose modulates vitamin C transport in adult human small intestinal brush border membrane vesicles. *J Nutr* 130:63-69.

11. Sabry, J.H., Fisher, K.H., and Dodds, M.L. 1958. Human utilization of dehydroascorbic acid. *J Nutr* 64:457-466.

12. Jacob, R.A., Spinozzi, G.M., Simon, V.A., Kelley, D.S., Prior, R.L., Hess-Pierce, B., and Kader, A.A. 2003. Consumption of cherries lowers plasma urate in healthy women. *J Nutr* 133:1826-1829.

13. Wells, W.W., and Xu, D.P. 1994. Dehydroascorbate reduction. *J Bioenerg Biomembr* 26:369-377.

14. Lykkesfeldt, J. 2002. Increased oxidative damage in vitamin C deficiency is accompanied by induction of ascorbic acid recycling capacity in young but not mature guinea pigs. *Free Radic Res* 36:567-574.

15. Welch, R.W., Bergsten, P., Butler, J.D., and Levine, M. 1993. Ascorbic acid accumulation and transport in human fibroblasts. *Biochem J* 294 (Pt 2):505-510.

16. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U.V., Chen, X.Z., Wang, Y., Brubaker, R.F., and Hediger, M.A. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 399:70-75.

17. Corpe, C.P., Tu, H., Eck, P., Wang, J., Faulhaber-Walter, R., Schnermann, J., Margolis, S., Padayatty, S., Sun, H., Wang, Y., et al. 2010. Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice. *J Clin Invest* 120:1069-1083.

18. Timpson, N.J., Forouhi, N.G., Brion, M.J., Harbord, R.M., Cook, D.G., Johnson, P., McConnachie, A., Morris, R.W., Rodriguez, S., Luan, J., et al. 2010. Genetic variation at the SLC23A1 locus is associated with circulating concentrations of L-ascorbic acid (vitamin C): Evidence from 5 independent studies with >15,000 participants. *American Journal of Clinical Nutrition* 92:375-382.

19. Eck, P., Erichsen, H.C., Taylor, J.G., Yeager, M., Hughes, A.L., Levine, M., and Chanock, S.J. 2004. Comparison of the genomic structure and variation in the two human sodium-dependent vitamin C transporters, SLC23A1 and SLC23A2. *Human Genetics* 115:285-294.

20. Erichsen, H.C., Eck, P., Levine, M., and Chanock, S. 2001. Characterization of the genomic structure of the human vitamin C transporter SVCT1 (SLC23A2). *Journal of Nutrition* 131:2623-2627.

21. Rumsey, S.C., Kwon, O., Xu, G.W., Burant, C.F., Simpson, I., and Levine, M. 1997. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem* 272:18982-18989.

22. Wu, X., and Freeze, H.H. 2002. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80:553-557.

CHAPTER 2

LITERATURE REVIEW

2.1 INFLAMATORY BOWEL DISEASE: PREVALENCE, MECHANISMS OF DISEASE

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), are chronic, relapsing, immune-mediated intestinal inflammation (1). The etiology of IBD is not fully understood, however, it has been extensively studied in the past few decades. The epidemiology of IBD has a wide range of variation around the world, with rising rates in many regions, associated with industrialization of nations (2). As such, Europe and North America have the highest incidence rates of both UC (24.3 per 100,000 person-years and 19.2 per 100,000 person-years, respectively) and CD (12.7 per 100,000 person-years and 20.2 per 100,000 person-years, respectively) (2). In Canada, IBD has a great burden on the Health Care System with economic cost ($2.8 billion in 2012) as well as total direct medical cost ($1.2 billion in 2012) (3).

Crohn's disease and ulcerative colitis have similar final common pathways; however, they are considered heterogeneous diseases because of various genetic, phenotypic, bacteriologic, immunologic, and therapeutic characterizations (1). In each IBD subtype, the chronic intestinal inflammation can be triggered by environmental factors that transiently rupture the mucosal barrier and aggravate immune responses to a subset of commensal enteric bacteria. Various genetic irregularities can lead to similar disease phenotypes, through causing defects in mucosal barrier function, immunoregulation or bacterial clearance (1).

2.2 INFLAMMATORY BOWEL DISEASES AND OXIDATIVE STRESS

It is now proposed that oxidative stress is a major pathogenic factor of IBD which drive fundamental cellular/tissue destructive mechanisms through an excessive release of reactive oxygen species (ROS) (4, 5). Several reports (6-9), assessing ROS levels in colonic biopsy specimens of individuals with IBD, consistently show that compared with normal mucosa from healthy controls, ROS production is considerably increased in the IBD group and that excessive production of ROS is responsible for oxidative damage in the gut of individuals with IBD (10-12). In individuals with IBD disrupted intestinal barrier and abnormal immunological events initiate an inflammatory cascade which begins with massive infiltration of leukocytes into the intestinal wall and release of ROS (13). The released ROS induce tissue damage and result in further recruitment of inflammatory cells, thus sustaining the inflammatory cascade and compromising cell integrity and function (13, 14). ROS also enhances intestinal inflammation through its profound effect on the expression of a variety of immune molecules, promoting neutrophil adherence, and increasing mucosal and vascular permeability (5, 15, 16). Obviously, it is an absolute necessity for cells and tissues to carefully regulate ROS levels via their elaborate antioxidant defense, particularly under inflammatory conditions such as IBD.

2.3 VITAMIN C AND INFLAMATION

Vitamin C is an essential dietary antioxidants with a significant role in regulation of ROS, and therefore, in initiation and perturbation of IBD (17). Ascorbate (ASC) is the functional form of vitamin C serves as a one-electron donor to produce ASC free radical which within cells is reduced back to ASC by nicotinamide adenine dinucleotide (NADH-) and nicotinamide adenine

dinucleotide phosphate (NADPH-) dependent reductases enzymes (18, 19). In inflammatory conditions such as IBD, when the production of ASC free radical exceeds the capacity of the enzymes, two molecules of ASC free radical react and form one molecule of ASC and one molecule of dehydrascorbic acid (DHA) which is the two-electron-oxidized form of ASC (19). DHA can't be detected in human blood because it is very unstable with a half-life about 2-6 minutes (20). Within cells, DHA is immediately recycled back to ASC by different mechanisms including direct reduction by glutathione (GSH) and enzymatic reduction by NADPH-dependent reductases (21).

During inflammatory conditions in the extracellular space, ASC oxidized to DHA which is absorbed into surrounding cells and immediately reduced back to ASC (22), the continuous mechanism within the cellular cytoplasm which maintains adequate levels of ASC to quench free radicals (23-25). Intracellular reduction of DHA to ASC results in efflux of ASC to extracellular fluids, as reported for endothelial cells, erythrocytes, and hepatocyte-like HepG cells (26, 27). Efflux of ASC to the blood from enterocytes and renal tubular cells is the fundamental mechanism for intestinal absorption and renal reabsorption of vitamin C, however, the responsible transporter and the mechanism for efflux of ASC is not known yet.

2.4 VITAMIN C: REGULATION, TRANSPORT

Most mammals synthesize vitamin C from glucose; however, humans, guinea pigs, and bats rely completely on dietary supply because of the lack of the *Gulo* gene, encoding L-gulono-lactone oxidase enzyme which is required for catalyzing the final step in vitamin C biosynthesis (28, 29).

In humans, plasma concentration following oral dosage of vitamin C is reflected in a saturation curve which is tightly regulated with a peak around 200 μmol/L and reaching a steady-state of 70–85 μmol/L, defining plasma saturation even after ingestion of an excessive amounts of vitamin C (3 g) (30, 31). At dosage above plasma saturation, oral bioavailability decreased and urinary excretion is increased which result in maintaining steady-state equilibrium (30). A steep decline in vitamin C bioavailability following ingestion of augmented oral doses suggests that intestinal transport is a key factor in sustaining whole body vitamin C homeostasis (32).

The tight regulation of vitamin C homeostasis is mainly controlled by different mechanisms, including active transport, facilitated diffusion transport and recycling (33). More important are particular mechanisms of transport and metabolism that deliberate vitamin C intracellularly to enhance its function as an antioxidant and an enzyme cofactor (33). ASC, the ionized form of vitamin C, is actively transported trough sodium dependent transporters; however, transport of DHA, the oxidized form of vitamin C, occurs via some members of facilitative glucose transporters. The facilitated diffusion of DHA along a concentration gradient, is sustained by immediate reduction of DHA to ASC after crossing the cell membrane (22, 34, 35), keeping the intracellular concentration of DHA sufficiently low.

Various vitamin C transporters and their encoding genes have been exclusively reviewed in following chapter **(Chapter 3, Manuscript 1)**.

2.5 ALTERNATIVE SPLICING IN VITAMIN C TRANSPORTER GENES

The first step in description of a transporter is to visit the structure of its responsible gene and to identify its splice variants. The concurrent and regulated molecular mechanisms of intron and exon characterization are commonly responsible for the splicing configuration in a certain transcript variant (36). Alternative splicing occurs through the regulated selection of different combinations of exons for inclusion into the mRNA to produce multiple mRNA and protein isoforms (37). Alternative splicing is involved in numerous human diseases and the process depends upon the tissue, developmental stage and disease versus normal conditions of the cell (38, 39). The majority of human genes (60–75%) are alternatively spliced (40, 41). Function of the gene is an important indicator for the number of alternative splicing, meaning that genes that are involved in cell communication, transcription regulation, and enzyme regulation are more commonly subject to alternative splicing (40, 42). As such, vitamin C transporters may consider potent candidate genes for alternative splicing.

2.5.1 ALTERNATIVE SPLICING AND ITS REGULATION

Alternative splicing is a fundamental process regulating eukaryotic gene through which different combinations of exons from the RNA precursor are spliced to be included in the mature mRNA (39, 43). It is usually postulated as the main mechanism to produce diverse proteins with different and even sometimes antagonistic functional and structural properties from a somehow limited set of protein coding genes (38, 44).

The phenomenon of alternative splicing was discovered first in the late 1970s, however, the sequencing of the human genome was the revolutionary time to capture the attention to the extent and importance of alternative splicing of pre-mRNA as a RNA regulatory mechanism for modulating the gene and protein content in the cell (37).

2.5.2 ALTERNATIVE SPLICING EVENTS VOCABULARY

The concurrent and regulated molecular mechanisms of intron and exon characterization are commonly responsible for the splicing configuration in a certain transcript variant (36). The formalization of the alternative splicing are classically described in 5 kinds of splicing events: exon skipped (ES), mutually exclusive exons (MA) (also called cryptic exons), intron retention (IR) (also called intron inclusion), alternative acceptor splice site (AA), and alternative donor splice site (AD) (38, 42). ES are exons that are included in reference form, but not included to the variants(42). On the other hand, MA are exons that are not included in the reference form, but are included in transcripts upon specific regulatory events or sequence mutations (42). Moreover, a plethora of variations in alternative splicing has been proposed which include various combinations that involve multiple instances of these classical events (38).

2.5.3 IDENTIFICATION OF ALTERNATIVE SPLICE VARIANTS

Most studies aim to identify splice variants of a certain gene, use the genome sequence of the gene as a reference onto which a variety of other types of information are projected including protein domain models, transcript sequences (full-length cDNA and expressed sequence tags

(ESTs)), the genome sequences of other species, and expression information from exon–exon junction microarrays (42).

Transcript sequences with full-length cDNA sequences are probably the most reliable method for identifying splice variations (42, 45). In this method, full length cDNA is mapped to its respective genomic DNA and alternative splicing events are determined based on analyzing clusters of overlapping transcripts (42). The advantage of using the complete transcript of cDNA is more accurate identification of the transcription start site, the splice sites, and the polyadenylation signal which were used to create the mature mRNA(42). However, this method has the disadvantage of the limited number of available full-length cDNAs, especially compared with EST data (42).

2.6 REFERENCES

1. Sartor, R.B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 3:390-407.

2. Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., et al. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 142:46-54 e42; quiz e30.

3. Crohn's and Colitis Foundation of Canada (CCFC). 2012. The Impact of Inflammatory Bowel Disease in Canada: 2012 Final Report and Recommendations. http://content.yudu.com/A200bd/2012ImpactReportEN/resources/2.htm.

4. McCord, J.M. 2000. The evolution of free radicals and oxidative stress. *Am J Med* 108:652-659.

5. Kruidenier, L., and Verspaget, H.W. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 16:1997-2015.

6. Keshavarzian, A., Sedghi, S., Kanofsky, J., List, T., Robinson, C., Ibrahim, C., and Winship, D. 1992. Excessive production of reactive oxygen metabolites by inflamed colon: analysis by chemiluminescence probe. *Gastroenterology* 103:177-185.

7. Sedghi, S., Fields, J.Z., Klamut, M., Urban, G., Durkin, M., Winship, D., Fretland, D., Olyaee, M., and Keshavarzian, A. 1993. Increased production of luminol enhanced chemiluminescence by the inflamed colonic mucosa in patients with ulcerative colitis. *Gut* 34:1191-1197.

8. Lih-Brody, L., Powell, S.R., Collier, K.P., Reddy, G.M., Cerchia, R., Kahn, E., Weissman, G.S., Katz, S., Floyd, R.A., McKinley, M.J., et al. 1996. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 41:2078-2086.

9. Simmonds, N.J., Allen, R.E., Stevens, T.R., Van Someren, R.N., Blake, D.R., and Rampton, D.S. 1992. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* 103:186-196.

10. Gaginella, T.S., Kachur, J.F., Tamai, H., and Keshavarzian, A. 1995. Reactive oxygen and nitrogen metabolites as mediators of secretory diarrhea. *Gastroenterology* 109:2019-2028.

11. McKenzie, S.J., Baker, M.S., Buffinton, G.D., and Doe, W.F. 1996. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest* 98:136-141.

12. Kruidenier, L., and Verspaget, H.W. 1998. Antioxidants and mucosa protectives: realistic therapeutic options in inflammatory bowel disease? *Mediators Inflamm* 7:157-162.

13. Keshavarzian, A., Banan, A., Farhadi, A., Komanduri, S., Mutlu, E., Zhang, Y., and Fields, J.Z. 2003. Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease. *Gut* 52:720-728.

14. Harris, M.L., Schiller, H.J., Reilly, P.M., Donowitz, M., Grisham, M.B., and Bulkley, G.B. 1992. Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon? *Pharmacol Ther* 53:375-408.

15. Siemankowski, L.M., Morreale, J., and Briehl, M.M. 1999. Antioxidant defenses in the TNF-treated MCF-7 cells: selective increase in MnSOD. *Free Radic Biol Med* 26:919-924.

16. Grisham, M.B. 1994. Oxidants and free radicals in inflammatory bowel disease. *Lancet* 344:859-861.

17. Aghdassi, E., Wendland, B.E., Steinhart, A.H., Wolman, S.L., Jeejeebhoy, K., and Allard, J.P. 2003. Antioxidant vitamin supplementation in Crohn's disease decreases oxidative stress. a randomized controlled trial. *Am J Gastroenterol* 98:348-353.

18. Wakefield, L.M., Cass, A.E., and Radda, G.K. 1986. Electron transfer across the chromaffin granule membrane. Use of EPR to demonstrate reduction of intravesicular ascorbate radical by the extravesicular mitochondrial NADH:ascorbate radical oxidoreductase. *J Biol Chem* 261:9746-9752.

19. Harrison, F.E., and May, J.M. 2009. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. *Free Radic Biol Med* 46:719-730.

20. Koshiishi, I., Mamura, Y., Liu, J., and Imanari, T. 1998. Degradation of dehydroascorbate to 2,3-diketogulonate in blood circulation. *Biochim Biophys Acta* 1425:209-214.

21. Wells, W.W., and Xu, D.P. 1994. Dehydroascorbate reduction. *J Bioenerg Biomembr* 26:369-377.

22. Washko, P.W., Wang, Y., and Levine, M. 1993. Ascorbic acid recycling in human neutrophils. *J Biol Chem* 268:15531-15535.

23. Lykkesfeldt, J. 2002. Increased oxidative damage in vitamin C deficiency is accompanied by induction of ascorbic acid recycling capacity in young but not mature guinea pigs. *Free Radic Res* 36:567-574.

24. Buettner, G.R. 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys* 300:535-543.

25. Linster, C.L., and Van Schaftingen, E. 2006. Glucuronate, the precursor of vitamin C, is directly formed from UDP-glucuronate in liver. *FEBS J* 273:1516-1527.

26. Upston, J.M., Karjalainen, A., Bygrave, F.L., and Stocker, R. 1999. Efflux of hepatic ascorbate: a potential contributor to the maintenance of plasma vitamin C. *Biochem J* 342 (Pt 1):49-56.

27. Mendiratta, S., Qu, Z.C., and May, J.M. 1998. Erythrocyte ascorbate recycling: antioxidant effects in blood. *Free Radic Biol Med* 24:789-797.

28. Nishikimi, M., Fukuyama, R., Minoshima, S., Shimizu, N., and Yagi, K. 1994. Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. *J Biol Chem* 269:13685-13688.

29. Nandi, A., Mukhopadhyay, C.K., Ghosh, M.K., Chattopadhyay, D.J., and Chatterjee, I.B. 1997. Evolutionary significance of vitamin C biosynthesis in terrestrial vertebrates. *Free Radic Biol Med* 22:1047-1054.

30. Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R.W., Washko, P.W., Dhariwal, K.R., Park, J.B., Lazarev, A., Graumlich, J.F., King, J., et al. 1996. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci U S A* 93:3704-3709.

31. Padayatty, S.J., Sun, H., Wang, Y., Riordan, H.D., Hewitt, S.M., Katz, A., Wesley, R.A., and Levine, M. 2004. Vitamin C pharmacokinetics: implications for oral and intravenous use. *Ann Intern Med* 140:533-537.

32. Lindblad, M., Tveden-Nyborg, P., and Lykkesfeldt, J. 2013. Regulation of vitamin C homeostasis during deficiency. *Nutrients* 5:2860-2879.

33. Wilson, J.X. 2005. Regulation of vitamin C transport. *Annu Rev Nutr* 25:105-125.

34. Rumsey, S.C., Kwon, O., Xu, G.W., Burant, C.F., Simpson, I., and Levine, M. 1997. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem* 272:18982-18989.

35. Rumsey, S.C., Daruwala, R., Al-Hasani, H., Zarnowski, M.J., Simpson, I.A., and Levine, M. 2000. Dehydroascorbic acid transport by GLUT4 in Xenopus oocytes and isolated rat adipocytes. *J Biol Chem* 275:28246-28253.

36. Fox-Walsh, K.L., Dou, Y., Lam, B.J., Hung, S.P., Baldi, P.F., and Hertel, K.J. 2005. The architecture of pre-mRNAs affects mechanisms of splice-site pairing. *Proc Natl Acad Sci U S A* 102:16176-16181.

37. Sammeth, M., Foissac, S., and Guigo, R. 2008. A General Definition and Nomenclature for Alternative Splicing Events. *Plos Computational Biology* 4.

38. Sammeth, M., Foissac, S., and Guigo, R. 2008. A general definition and nomenclature for alternative splicing events. *PLoS Comput Biol* 4:e1000147.

39. Black, D.L. 2003. Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem* 72:291-336.

40. Johnson J, C.J., Garrett-Engele P, Kan Z, Loerch P, Armour C, Santos R, Schadt E, Stoughton R, Shoemaker D. . 2003. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* 302:2141-2144.

41. Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. 2001. Initial sequencing and analysis of the human genome. *Nature* 409:860-921.

42. Zavolan, M., and van Nimwegen, E. 2006. The types and prevalence of alternative splice forms. *Curr Opin Struct Biol* 16:362-367.

43. Lopez, A.J. 1998. Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation. *Annu Rev Genet* 32:279-305.

44. Florea, L., Di Francesco, V., Miller, J., Turner, R., Yao, A., Harris, M., Walenz, B., Mobarry, C., Merkulov, G.V., Charlab, R., et al. 2005. Gene and alternative splicing annotation with AIR. *Genome Res* 15:54-66.

45. Imanishi, T., Itoh, T., Suzuki, Y., O'Donovan, C., Fukuchi, S., Koyanagi, K.O., Barrero, R.A., Tamura, T., Yamaguchi-Kabata, Y., Tanino, M., et al. 2004. Integrative annotation of 21,037 human genes validated by full-length cDNA clones. *PLoS Biol* 2:e162.

CHAPTER 3

MANUSCRIPT 1

This manuscript is in press by the Journal of Advances in Nutrition

(Invited first author, by the journal board of editors)

GENETIC VARIATION IN HUMAN VITAMIN C TRANSPORTER GENES IN COMMON COMPLEX DISEASES

Mandana Amir Shaghaghi, Olena Kloss, Peter Eck

Afflications: Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, R3T 2N2.

3.1 ABSTRACT

Adequate plasma, cellular, and tissue vitamin C concentrations are required for maintaining optimal health, through suppression of oxidative stress and optimizing function of certain enzymes which require vitamin C as a co-factor. Polymorphisms in the vitamin C transporter genes, compromising genes encoding sodium-dependent ascorbate (ASC) transport proteins and also genes encoding facilitative transporters of dehydroascorbic acid (DHA), are associated with plasma and tissue cellular ascorbate status and hence cellular redox balance. This review summarizes our current knowledge of the links between variations in vitamin C transporter genes and common chronic diseases. We conclude that emerging genetic knowledge has a good likelihood of defining future personalized dietary recommendations and interventions; however, further validations through biological studies as well as controlled dietary trials are required to identify predictive and actionable genetic biomarkers. We further advocate the need to consider genetic variation of vitamin C transporters in future clinical and epidemiological studies on common complex disease.

3.2 INTRODUCTION

Vitamin C is an essential nutrient and the most important plasma water-soluble antioxidant which plays critical roles in biosynthesis of neurotransmitters and collagen, absorption of nonheme iron, detoxification of exogenous compounds and cytochrome P-450 activity, and regulation of hypoxia-inducible factor 1α (HIF-1α) (1, 2). In addition, it plays a major role as an antioxidant and free radical scavenger and protects against lipid peroxidation (3). Vitamin C has also been shown to function in sparing or reconstituting vitamin E for protection of lipid

membranes (4, 5). Therefore, maintaining adequate plasma and tissue cellular vitamin C concentrations is crucial for normal metabolic function of the body and preventing many common complex diseases (6-14).

Epidemiological studies show that individuals with reduced plasma vitamin C concentrations display an elevated risk of different chronic diseases (6). A review (15) of data from more than 90 epidemiologic studies which related the dietary intake of vitamin C to various types of cancer (breast, oral, gastric, esophageal, pancreatic, lung, cervical, and rectal) revealed a negative correlation in three-fourths of the studies. Besides, each 20 μM increase in plasma vitamin C concentrations is associated with a 20% reduced risk of all-cause mortality (16) and a 9% relative decline in risk of heart failure (17).

A marginal vitamin C deficit (11 μM < plasma concentration < 24 μM) was estimated to affect up to 10% of adults in industrialized countries (13, 18, 19). Although vitamin C status is mainly determined by the dietary intake, it should be noted that a complex interplay of intrinsic metabolic factors, such as oxidative stress, inflammation, recycling, and transmembrane transport contribute to the metabolic turnover and therefore vitamin C status (20). The metabolic turnover can be impacted by genetic variations and thus vitamin C status could be impaired even at dietary intake concentrations which are currently regarded as adequate for the general population, if an individual carries a rare allele.

Transporters of the different forms of vitamin C directly regulate vitamin C intracellular bioavailability (**Figure 3.1A**). The elimination of selected ascorbic acid transporters in the mouse

results in severely impacted pharmacokinetics and reduced offspring viability (31) or even total offspring lethality (32). Therefore, variations in genes of the human vitamin C transporter pathways may impact on disease development and outcomes (**Figure 3.1B**). This review summarizes existing knowledge the variations in vitamin C transporter genes and disease associations. Two vitamin C transmembrane pathways are distinct by their substrates, where SLC23A1 and SLC23A2 mediate ascorbic acid transport, while dehydroascorbic acid is shuttled by the four members of the facilitative glucose transporter family GLUT1 (*SLC2A1*), GLUT2 (*SLC2A2*), GLUT3 (*SLC2A3*), GLUT4 (*SLC2A4*) which will all be reviewed.

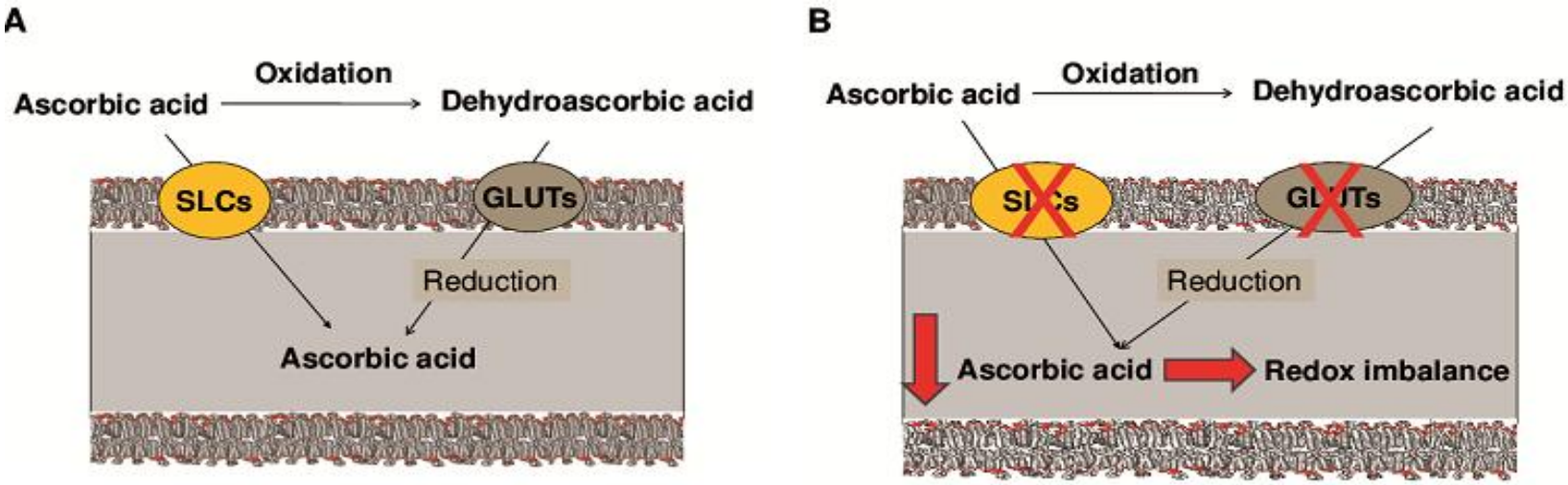


Figure 3.1

A) General schematic for cellular uptake of vitamin C, The concentration of ascorbic acid in the intracellular environment is tightly controlled through regulation of the transporters. Ascorbic acid (ASC) is the functional form of vitamin C which transported into the cell trough sodium dependent ascorbic acid transporter family (SLCs). SLCs are comprised of SLC23A1 and SLC23A2 which have specific cell expression and precise sub-cellular localization. The SLC23A1 protein is responsible for active transport of ASC from the epical luminal surface of the intestinal tract and kidney (21, 22). The SLC23A2 protein is expressed in most human tissues, except lung and skeletal muscle (22, 23), is thought to regulate intracellular concentrations of ASC for subsequent protection of the cell from oxidative stress as well as promotion the maturation of type I collagen. Dehydroascorbic acid (DHA), the oxidized form of ASC, is transported into the cell by some members of facilitative glucose transporter family (GLUTs) including GLUT1 (SLC2A1), GLUT2 (SLC2A2), GLUT3 (SLC2A3) (24-27) which have specific cell expression and transport activity. Within cells, DHA is immediately recycled back to ASC and this process sustain the intracellular ASC and therefore redox balance (28-30).
B) Proposed mechanism for association between variations in vitamin C transporters and common complex disease, genetic variation in each ascorbic acid transporters (SLCs) or dehydroascorbic acid transporters (GLUTs) could modulate transport of ASC or DHA thereby resulting in reduced intracellular Vitamin C, redox imbalance, and thus increased risk of common complex disease.

3.3 CURRENT STATUS OF KNOWLEDGE

3.3.1 SODIUM-DEPENDENT VITAMIN C TRANSPORTERS

The active transport of ASC across cell membrane is generated by two sodium-dependent ascorbate transporters which were first cloned in 1999 (33). The two transporters, SLC23A1 and SLC23A2 , mediate sodium and energy dependent ASC transport against a concentration gradients into cells, resulting in intracellular concentrations which can be 50 fold higher than the extracellular fluids (33, 34). SLC23A1 and SLC23A2 co-transport Na^+ and ASC with a 2:1 stoichiometry, utilizing the electrochemical Na^+ gradient (35, 36).

The SLC23A1 and SLC23A2 are responsible for the maintenance of vitamin C concentrations in nearly all cells (except erythrocytes), tissues, and extracellular fluids (20). Genetic pattern of both *SLC23A1* and *SLC23A2* share common intron/exon borders and have related coding sequence, however the genes differ 10-fold in size (16 kb vs. 160 kb, respectively) and in linkage disequilibrium (37). The encoded proteins of the two transporters are comparable in amino acid sequence and structure, however, they have different tissue distributions (33, 37).

SLC23A1 expression is confined to epithelia, such as intestinal, renal and hepatic tissues (33, 38) and it has the major role in whole body ascorbate homeostasis, through its function as sole apical ascorbic acid transporter in the proximal renal epithelial cell (31). SLC23A1 has low affinity [Michaelis constant (Km)[4] of 65–252 μM)] (21) and high capacity (Vmax of approximately 15 pmol/min/cell) (39), establishing the ability of this transporter to maintain the whole-body homeostasis (21, 22).

The *SLC23A1* locus on human chromosome 5q31.2 contains 16 exons (37, 40), spanning about 17.3 kilobases. A total of 1440 variations are listed in the Single Nucleotide Polymorphism Database (dbSNP), of which 294 locate to the coding region (187 missense, 91 synonymous, 11 frameshift, 4 insertions). Many of the variations in *SLC23A1* have not been verified in different populations, such as the HapMap cohorts (41) and the majority of the variations are neither reported in the literature nor functionally characterized. Genetic linkage throughout the locus is high with some evidence of linkage blocks in the 5' and 3' of the gene (37). Variations in *SLC23A1* seem to affect the vitamin C status, but current evidence remain inconclusive (42).

SLC23A2 is distributed in cells of most tissues (33) and contributes to delivering vitamin C into cells for some metal ion-dependent enzymatic reactions as well as protecting cells from oxidative stress (33, 43, 44). SLC23A2 has low capacity (approximately 1 pmol/min/cell) (21, 39) and high affinity (*K*m-values of 8–69 μM) (21, 35, 45) for ASC transport, mediating uptake of ASC by cells of peripheral organs from the extracellular fluid (22, 23). A difference in membrane epithelial cells distribution of SLC23A1 and SLC23A2 suggests non-redundant functions for these two transporters (46, 47).

The *SLC23A2* locus on human chromosome 20p13 contains 17 exons (37), spanning about 160 kilobases, and is roughly ten times bigger than *SLC23A1*. A total of 8165 variations are listed in dbSNP, of which 262 locate to the coding region (138 missense, 120 synonymous, 4 frameshift). Many of the variations in *SLC23A2* have not been verified in different populations, such as the HapMap cohorts (41) and the majority of these variations are neither reported in the literature nor functionally characterized. Genetic linkage throughout the locus is moderate (37), however,

linkage blocks are not defined (37). Variations in *SLC23A2* are jet to be reported to affect the vitamin C status.

When the patterns of SNPs in *SLC23A1* and *SLC23A2* were compared, a significant number of the SNPs in *SLC23A1* were population-specific in either Caucasians or African Americans, including four nonsynonymous SNPs; however, nearly all SNPs in *SLC23A2* are shared between the two population, African Americans and Caucasians (48). It was deduced that the *SLC23A1* gene does tolerate variations better than *SLC23A2*, indicating a higher physiological importance for the latter.

3.3.2 POLYMORPHISMS IN SODIUM-DEPENDENT VITAMIN C TRANSPORTERS AND PATHOLOGICAL RELEVANCE

The risk association of several SNPs in *SLC23A1* and *SLC23A2* genes with a variety of common chronic diseases including various cancer (49-55), inflammatory bowel disease (56), preterm delivery (57), coronary heart disease (58), and optic neuropathy (59, 60) have been evaluated **(TABLE3.1)**.

Table 3.1: Phenotype-genotype association of single-nucleotide polymorphisms (SNPs) in the human sodium dependent ascorbate transporter genes with chronic diseases

Gene	SNP	Allele (Major/ minor)	Location	Disease	Population	Sample size (Case/control)	Findings	Reference
SLC23A1	rs11950646	A/G	chr5: 139378785	Follicular lymphoma	USA	1292/1375	↑risk GG genotype	Skibola et al., 2008 (49)
				Small lymphocytic lymphoma/ Chronic lymphocytic leukemia	Germany	494/494	↑risk GG genotype	Skibola et al., 2008 (49)
	rs6596473	G/C	chr5: 139374887	Follicular lymphoma	USA	1292/1375	↑risk CC genotype	Skibola et al., 2008 (49)
				Chronic lymphocytic leukemia Diffuse large B-cell lymphoma	Germany	494/494	↑Chronic lymphocytic leukemia risk ↓Diffuse large B-cell lymphoma risk CC genotype	Skibola et al., 2008 (49)
				Lower concentration of ocular ascorbate	India	60/-	↑risk C-carrier	Senthilkumari et al., 2014 (60)
	rs10063949	A/G	chr5: 139383837	Inflammatory bowel disease	Canada	311/142	↑ Crohn's disease risk G-carrier	Amir Shaghaghi et al., 2014 (56)
SLC23A2	rs6133175	A/G	chr20: 4911113	Non-Hodgkin lymphoma-Diffuse large B-	USA	1292/1375	↑risk GG genotype	Skibola et al., 2008 (49)

				cell lymphoma-Small lymphocytic lymphoma				
	rs1715364	T/C	chr20: 4918250	Small lymphocytic lymphoma-Diffuse large B-cell lymphoma	USA	1292/1375	↑risk CC genotype	Skibola et al., 2008 (49)
				Small lymphocytic lymphoma-Diffuse large B-cell lymphoma	Germany	494/494	↑risk CC genotype	Skibola et al., 2008 (49)
	rs1715385	G/A	chr20: 4907024	Non-Hodgkin lymphoma-Diffuse large B-cell lymphoma	USA	1292/1375	↑risk AA genotype	Skibola et al., 2008 (49)
	rs1776948	G/A	chr20: 4950467	Non-Hodgkin lymphoma-follicular lymphoma-small lymphocytic lymphoma	USA	1292/1375	↑risk AA genotype	Skibola et al., 2008 (49)
				Non-Hodgkin lymphoma-follicular lymphoma	Germany	494/494	↑risk AA genotype	Skibola et al., 2008 (49)
	rs6139587	T/A	chr20: 4961828	Non-Hodgkin lymphoma - Small lymphocytic	USA	1292/1375	↑risk AA genotype	Skibola et al., 2008 (49)

				lymphoma				
	rs4987219	G/C	chr20: 4884300	Colorectal adenoma	USA	656/665	↓risk C-carrier	Erichsen et al., 2008 (50)
				Human papilloma virus-Head and neck squamous cell carcinomas association	USA	319/495	↑risk G-carrier	Chen et al., 2009 (51)
				Esophageal squamous cell carcinoma	Japan	49/-	↑risk leukopenia C-carrier	Minegaki et al., 2014 (52)
	rs1110277	T/C	chr20: 4874036	Colorectal adenoma	USA	656/665	↓risk C-carrier	Erichsen et al., 2008 (50)
		C/T		Esophageal squamous cell carcinoma	Japan	49/-	↑risk stomatitis T-carrier	Minegaki et al., 2014 (52)
	rs12479919	C/T	chr20: 5000094	Gastric cancer	Poland	279/414	↓risk TT genotype	Wright et al., 2009 (53)
				Bladder cancer	USA	832/1191	↑risk CT genotype	Andrew et al., 2009 (55)
				Lower concentration of ocular ascorbate	India	60/-	↑risk TT genotype	Senthilkumari et al., 2014 (60)
	rs6116569	C/T	chr20: 4884071	Gastric Cancer	European	365/1284	↑risk T- carrier	Duell et al. 2013 (54)
	rs2681116	G/A	chr20: 4970685	Preterm delivery	USA	271/572	↑risk GA genotypes	Erichsen et al., 2006 (57)

	rs6139591	C/T	chr20: 4970713	Preterm delivery	USA	271/572	↑risk T-carrier	Erichsen et al., 2006 (57)
				Acute coronary syndrome	Denmark	936/1580	↑risk TT genotype	Dalgard et al., 2013 (58)
	rs1776964	C/T	chr20: 4880308	Preterm delilvery	USA	271/572	↓risk TT genotype	Erichsen et al., 2006 (57)
				Acute coronary syndrome	Denmark	936/1580	↑risk TT genotype	Dalgard et al., 2013 (58)
	rs1279383	A/G	chr20: 5002446	Primary open glaucoma	Mediterranean	150/150	↑risk GG genotype	Zanon-Moreno et al., 2011 (59)

3.3.2.1 Cancer

Vitamin C plasma and tissue concentrations have been postulated to affect relative cancer risk. The antioxidant effect of vitamin C may prevent cancers by inducing apoptosis and suppressing tumor cell growth (61-63) while counterbalancing DNA damage through scavenging of reactive oxygen species (64). Vitamin C also protects mucosal tissues from oxidative damage (65, 66) and plays an anti-tumorigenic role via sustaining proper collagen formation and matrix stabilization (67). As such, the risk association of vitamin C transporter genes with various intestinal cancers has been the key interest for several studies. In a study (50) with 656 patients with colorectal adenoma and 665 healthy controls, participants were genotyped for four SNPs in *SLC23A1* gene and 11 different SNPs in SLC23A2 gene. No association between common SNPs in *SLC23A1* and colorectal cancer was revealed. For *SLC23A2*, there was no association with SNPs, but the haplotype G-C (rs4987219 and rs1110277) was associated with a reduction in the risk of colorectal adenoma (50). In a study on gastric cancer (53), association between 13 genetic variants of the *SLC23A1* and *SLC23A2* genes with the disease was examined. Among the 13 SNPs examined, gastric cancer was inversely associated with one SNP (rs12479919) in *SLC23A2* gene, while no association with variants in *SLC23A1* gene was determined. Compared to rs12479919-G/G genotypes, homozygotes for the minor allele A/A had a lower risk of gastric cancer (53). In the aforementioned study, a haplotype in *SLC23A2* gene, containing the common allele of the rs6139591, rs2681116, and rs14147458 SNPs, was inversely associated with gastric cancer (53). Likewise, in another study with 365 patients with gastric cancer and 1,284 controls (54), the genotype rs6116569-C/T and the two haplotypes, CGTC (rs6052937, rs3787456, rs6116569, rs17339746) and ATC (rs6139587, rs6053005, rs2326576), in the in the *SLC23A2*

gene were associated with gastric cancer risk while no association was found with variants in *SLC23A1*.

Variants in vitamin C transporter genes have also been associated with other type of cancers. In a population based study with 832 patients with bladder cancer and 1,191 healthy controls (55), variant rs12479919-CT in *SLC23A2* has been identified as a high risk genotype for gene-gene effect on bladder cancer. Indeed, the interaction of *SLC23A2* (rs12479919) and *SCARB1*-rs4765621 (the gene scavenger receptor class B) showed the strongest effect on the higher risk of bladder cancer (55). In another study with 1,292 patients and 1,375 healthy controls (49), several SNPs in *SLC23A1* and *SLC23A2* have been associated with increased risk of Non-Hodgkin lymphoma. In this study, individuals with the *SLC23A1* genotypes rs6596473-C/C and rs11950646-G/G showed an 80% elevated risk of lymphoma. Moreover, several SNPs in *SLC23A2* (**Table 1**) as well as two haplotypes (AA: rs1776948, rs6139587 and AAC: rs1715385, rs6133175, rs1715364) in the gene were found to be associated with increased risk of the disease (49). Authors conclude that both vitamin C uptake and storage are involved in the pathogenesis of lymphoma (49).

Variation in the *SLC23A2* gene also affected the initiation or sustention of head and neck cancer in patients with human papillomavirus type 16 infection (HPV16) (51). In a study with 319 patients with head and neck cancer and 495 frequency-matched controls (51), the risk of the cancer associated with HPV16 was decreased among rs4987219-C/C homozygotes in the *SLC23A2* gene, compared to those with a wild-type allele. The authors suggest that the SNP modifies the risk of head and neck cancer associated with HPV16 infection through the role of

ASC in the maintenance of the epidermal barrier, maturation of type I procollagen, intracellular antioxidant, or its immunostimulatory effect (51).

Vitamin C transporter genes have not only been associated with increased risk of different types of cancer but also have been suggested as predictive biomarkers for therapies. In a study with 49 patients with Esophageal Squamous Cell Carcinoma (52), rs4987219 and rs1110277 in the *SLC23A2* gene showed correlation with severe toxicities (acute stomatitis and leucopenia) (**TABEL 3.1)**, after treatment with a Definitive 5-Fluorouracil/Cisplatin-Based Chemoradiotherapy (52).

3.3.2.2 Inflammatory bowel disease

In addition to associations with various intestinal cancers, a variation in ASC transporters is associated with inflammatory bowel disease (IBD), where oxidative damage plays a key role in initiation and progression of the disease (68). In a study with 311 people with IBD and 142 controls (56), the SNP rs10063949-G allele in the *SLC23A1* gene was associated with increased risk of Crohn's disease. Specifically, rs10063949-A/G heterozygotes had a 2.5-fold elevated risk of Crohn's disease while rs10063949-G/G homozygotes had a 4.7-fold elevated risk, compared to wild type homozygotes (56).

3.3.2.3 Pregnancy complications

Vitamin C deficiency (measured by dietary intake or ascorbic acid concentrations in serum, leukocytes, or cord blood) has been found in several epidemiologic investigations (14, 69-73) to be associated with premature rupture of membranes and preterm delivery (<37 weeks' gestation), a leading cause of neonatal mortality and morbidity (57). In view of the necessity of vitamin C for preservation of collagen and potency of membrane tensile (57, 69), genetic variants in *SLC23A1* and *SLC23A2* have also been associated to the risk of preterm delivery. Associations have been found between haplotypes in *SLC23A1* gene with spontaneous preterm delivery (57). Moreover, carrier of one or two minor alleles of variant rs6139591-T of the *SLC23A2* gene showed a 1.7-fold and a 2.7-fold higher risk for spontaneous preterm birth, respectively (57). Likewise, heterozygous individuals for rs2681116-G/A in *SLC23A2* showed 1.9 fold increased risk of preterm birth, however, analysis of the homozygous carrying minor alleles (rs2681116-A/A) showed no effects. The authors' speculate that the failure to detect significant association between rs2681116-A/A homozygous individuals with the risk of preterm delivery was related to small numbers of study population (57).

3.3.2.4 Coronary heart disease

Variations in *SLC23A2* have also been associated with acute coronary syndrome (58) where vitamin C is suggested to have cardioprotective influences, due to its anti-oxidative effects, and its beneficial effects on endothelial function and the collagen content of the atherosclerotic plaques (58, 74). A 5.4-fold elevated risk of acute coronary syndrome was observed (58) in women with the rs6139591-T/T genotype who had low intake of dietary vitamin C. Moreover,

women with the rs1776964-T/T genotype with high intake of vitamin C had a 3.4 fold increased risk of acute coronary syndrome, compared with C/C-homozygotes with low intake. Accordingly, the authors conclude that the effects of genotype may not be completely compensated by high dietary intake of vitamin C (58).

3.3.2.5 Optic neuropathy

Lack of vitamin C antioxidant capacity is also associated with glaucomatous optic neuropathy, where oxidative stress is related to the neuronal death (75, 76). Indeed, significant lower concentrations of vitamin C have been observed in plasma (59), normal-tension (77), and in the secondary aqueous humor (78) of glaucomatous patients. In a study among 150 patients with open-angle glaucoma and 150 controls (59), genotype rs1279386-G/G in *SLC23A2* was associated with higher risk of the disease (1.7 fold) as well as lower plasma vitamin C concentration (9.0±1.4 μg/ml versus 10.5±1.6 μg/ml, in patients and 10.9±1.6 μg/ml versus 12.1±1.8 μg/ml, in controls). In this study no association was found between polymorphisms in the *SLC23A1* gene with open-angle glaucoma (59). In a more recent study (60), polymorphisms in *SLC23A1* and *SLC23A2* genes were found to influence ASC concentration in aqueous humor and lens nucleus, in 60 patients undergoing small incision cataract surgery. SNPs rs6596473 in *SLC23A1*gene and rs12479919 in *SLC23A2* gene showed association with decreased ocular ascorbate concentration in carriers of the variant allele compared to the common homozygotes. For rs6596473 the per variant allele-C difference in aqueous humor ASC was -217 μmol/L, while for rs12479919 per variant allele-T difference in lens nucleus ASC was 0.085 μmol/G (60), compared to homozygotes common allele (G/G and C/C, respectively).

All the studies mentioned above, confirmed numerous minor frequency genotypes and haplotypes of the *SLC23A1* and *SLC23A2* genes, associated with various chronic diseases. Most findings were reported on an individual basis in cohorts of limited sizes. Therefore it is warranted to validate these findings in larger cohorts in order to utilize it as actionable biomarker of the respective common complex diseases.

3.3.3 FACILITATED DIFFUSION VITAMIN C TRANSPORTERS

DHA is one dietary source of vitamin C, beside ASC, which can be absorbed across the brush border membrane. Upon entry into the enterocyte, DHA is reduced either enzymatically or chemically back to ASC and thus maintains a concentration gradient, favoring DHA uptake (23, 27). Local DHA absorption may be especially important during intestinal inflammatory conditions, where the immune cells oxidative burst increases extracellular oxidation of ASC to DHA (23, 79). The produced DHA is transported into enterocytes or other bystander cells followed by immediate reduction to ASC and thus boosts intracellular concentrations of the free radical scavenger (28-30). In regard of whole body homeostasis, this might also prevent patients with chronic intestinal inflammation from becoming scorbutic (23, 27). Likewise, in any inflammatory condition throughout the body, where ASC gets oxidized to DHA in extracellular fluid, the produced DHA is taken up by specific facilitative diffusion transporters for various cells/tissues to elevate intracellular ASC (80-86).

SLC2A1 (GLUT1), SLC2A2 (GLUT 2), SLC2A3 (GLUT3), SLC2A4 (GLUT4), SLC2A8 (GLUT8) are the five facilitated DHA transporters identified (24-27). They are members of the SLC2A solute carriers' gene family, encoding for the GLUT proteins of facilitated sugar transporters. It is postulated that Vitamin C accumulation in cells occurs in part through transport of DHA by the carriers of the SLC2A family. It should be noted that DHA diffusion to some specific cell types is competitively inhibited by excessive glucose in plasma (24, 26). However, this inhibition might not be relevant in tubular cells of the kidney and on the luminal surface of absorptive intestinal epithelia (26, 45, 87, 88). Moreover, DHA diffusion into cells might be impeded during high glucose status through the lack of location of SLC2A transporters to the plasmalemma membrane.

The DHA-GLUT transporters show tissue and cell specific expression as well as various affinities and efficiencies in DHA transport (24, 26, 89, 90). SLC2A1 is expressed in a extensive variety of cells throughout the body with a particularly high expression in endothelial and epithelial-like barriers of the brain, peripheral nerve, eye, placenta and lactating mammary gland (24, 91, 92) and exhibits DHA transport activity defined by a Km of 1.1 mM and a Vmax of 108 pmol/min/oocyte (24). SLC2A2 is mainly expressed in brain, spleen, kidney, pancreas, liver, and the basolateral membranes of intestinal epithelial cells (90, 91, 93) and transports DHA with a *Km* of 2.33 mM and a *V*max of 25.9 pmol/min/oocyte (27). SLC2A3 is expressed particularly in the brain, neurons, and intestinal epithelial cells (24, 91) and has DHA transport activity defined by a Km of 1.7 mM and a Vmax of 241 pmol/min/oocyte (24). SLC2A4 is mainly found in adipose tissues as well as skeletal and cardiac muscle cells (26, 91) with a DHA transport activity showing a *Km* of 0.98 mM and a *V*max of 66 pmol/min/oocyte) (26). GLUT8 is

expressed in testis, blastocyst, brain, muscle and adipose tissues with DHA a transport activity defined by a *Km* of 3.23 mM and a *V*max of 10.1pmol/min/oocyte (27).

3.3.4 POLYMORPHISMS IN FACILITATIVE DIFFUSION VITAMIN C TRANSPORTERS AND PATHOLOGICAL RELEVANCE

Genetic variation in the DHA-GLUT transporter genes are associated with various common complex diseases, which could not only be attributed to disturbed disaccharide transport, but also to disturbed transport of alternative substrates, such as DHA. The linked between the diabetes related traits and impaired glucose metabolism are not the main focus of this section of the review. Our focus is to review the association studies with respect to DHA-GLUT variation and common complex disease, other than directly to diabetes related traits, e.g. fasting blood glucose. The number of these studies is relatively limited **(TABLE 3.2)**.

3.3.4.1 Diabetes complications

A variety of studies have found associations between variations in DHA-GLUT genes and diabetes related traits (99, 100, 102) as well as diabetes complications such as albuminuria (94), retinopathy (95, 107), and nephropathy (108), in which etiology might involve modulations to DHA transport. In regard to Vitamin C metabolism, excess of glucose during conditions of uncontrolled diabetes may competitively blocks uptake of DHA through facilitative GLUTs and thus impairs the transport of DHA by cells and impacts on the intracellular redox imbalance (23). As such, considering diabetes as a well established risk factors for CVD, in a study with 2383 incidence cases of CVD (fatal and nonfatal) (103), the contribution of 46 type 2 diabetes related

SNPs to CVD incidence was examined. Out of the 46 genetic variants examined, the variant rs11920090 in *SLC2A2* was significantly associated with incident CVD, independent of baseline diabetes status (103).

Table 3.2: Phenotype-genotype association of single-nucleotide polymorphisms (SNPs) in the human dehydroascorbic acid transporter genes with chronic diseases

Gene	SNP	Allele (Major /minor)	Location	Disease	Population	Sample size (Case/control)	Findings	Reference
SLC2A1	rs841847	C/T	chr1: 42937037	Diabetic albuminuria and macroalbuminuria	n_1=African American n_2=European American	n_1=2156/- n_2=8122/ 9453	↑risk TT genotype ($n_{2)}$	Hsu et al., 2010 (94)
	rs841846	A/G	chr 1: 42938000	Severe diabetic retinopathy	African American	473	↑risk (not specified)	Roy et al (95)
	rs3754218	G/T	chr1: 42933897	Renal cell carcinoma	England	92/99	↑ risk GT genotype	Page et al., 2005 (96)
	rs3820589	A/T	chr 1: 42960373	Renal cell carcinoma	England	92/99	↑ risk T-carrier	Page et al.,2005 (96)
	rs4658	C/G	chr 1: 42926579	Nonalcoholic Fatty Liver Disease	Spain	520/521	↑risk GG genotype	Vazquez-Chantada et al., 2013 (97)
	rs841856	G/T	chr 1: 42934442	Nonalcoholic Fatty Liver Disease	Spain	520/521	↑risk TT genotype	Vazquez-Chantada et al., 2013 (97)
	rs2229682	G/A	chr 1: 42929964	Spina bifida meningomyelocele	Hispanic and Caucasian, American	507/184	↑risk A-carrier	Davidson et al., 2008 (98)

SLC2A2	rs5393	C/A	chr 3: 171027131	Impaired glucose tolerance	Finland	259/248	↑risk AA genotype	Laukkanen et al., 2005 (99)
	rs5394	C/T	chr 3: 171027104	Impaired glucose tolerance	Finland	259/248	↑risk of type 2 diabetes T-carrier	Laukkanen et al., 2005 (99)
	rs5404	G/A	chr 3: 171007166	Impaired glucose tolerance	Finland	259/248	↑risk of type 2 diabetes A-carrier	Laukkanen et al., 2005 (99)
	rs5400	A/G	chr 3: 171014511	Impaired glucose tolerance	Finland	259/248	↑risk A-carrier	Laukkanen et al., 2005 (99)
				Type 2 diabetes	Finland	1170/ 983	↑risk GG genotype	Willer et al., 2007 (100)
				Prostate cancer	American	6642	↓ risk G- carrier	Meyer et al., 2010 (101)
	rs11920090	T/A	chr3: 170999732	Healthy individuals	European	76558/-	↑risk higher fasting glucode level and type 2 diabetes A-carrier	Dupuis et al., 2010 (102)

				History of CVD	Denmark	6049/- (Inter study)	↑risk A-carrier	Borglykke et al., 2012 (103)
				History of CVD	Denmark	9572/- (pooled analyses)	↑risk A-carrier	Borglykke et al., 2012 (103)
				History of CVD	Denmark	3523/- (Monica study)	↑risk A-carrier	Borglykke et al., 2012 (103)
	rs5398	T/C	chr3: 17099804	Negative mood delusions	n_1=German n_2= European American	n_1=927/2168 n_2=1247/1434	↑risk C-carrier	Meier et al., 2012 (104)
	rs1499821	A/G	chr 3: 172207423	Negative mood delusions	n_1=German n_2= European American	n_1=927/2168 n_2=1247/1434	↑risk G-carrier	Meier et al., 2012 (104)
	rs11924032	A/G	chr 3: 172217793	Negative mood delusions	n_1=German n_2= European American	n_1=927/2168 n_2=1247/1434	↑risk G-carrier	Meier et al., 2012 (104)
	rs9875793	A/G	chr 3: 170686573	Negative mood delusions, Bipolar disorder	n_1=German n_2= European American	n_1=927/2168 n_2=1247/1434	↑risk G-carrier	Meier et al., 2012 (104)
	rs8192675	G/A	chr 3: 171007094	Negative mood delusions	n_1=German n_2= European American	n_1=927/2168 n_2=1247/1434	↑risk A-carrier	Meier et al., 2012 (104)
				Hypertension	n_1=African Americans n_2= European	n_1=167 n_2=237	↓ High density lipoprotein A-carrier	Le et al., 2013 (105)

					Americans		(n_2)	
SLC2A4	rs5417	C/A	chr 17: 7281743	Obstructive sleep apnea syndrome	China	412/156	↑risk A-carrier	Yin et al., 2014 (106)
SLC2A5	rs5438	G/A	chr 1: 9069561	Hypertension	n_1=African Americans n_2 =European Americans	n_1=167 n_2=237	↑serum uric acid GA genotype (n_2)	Le et al., 2013 (105)

3.3.4.2 Cancer

Variants in DHA-GLUT genes have been proposed to have diverse effects on relative risk for renal and prostate cancers; however, the overall studies are limited (96, 101) with no observed association in one study (109). In a study with 92 patients with renal cell carcinoma and 99 healthy controls (96), carriers of the minor allele rs3820589-T as well as heterozygotes for rs3754218-G/T in *SLC2A1* showed higher incidences of renal cancer. On the other hand, in the Atherosclerosis Risk in Communities Study (ARIC) study with 6642 patients with prostate cancer (101), SNP rs5400-G in *SLC2A2* was associated with 24% lower cancer risk in Caucasians but not in African Americans. The authors suggest that, despite uncertainly about the mechanism involved in the observed association, *SLC2A2* may be involved in prostate cancer progression due to several reports linking several large scale duplications on chromosome 3q, the region containing *SLC2A2*, with prostate cancer.

3.3.4.3 Psychological disorders

Variants in the *SLC2A2* gene were found to be associated with bipolar disorder which is a severe psychiatric condition with fundamental and distinctive alteration in emotion regulation and perception (104). In a study with 2174 patients with bipolar disorder and 3601 healthy controls (104), the minor alleles for several variants in *SLC2A2* (rs5398-C, rs1499821-G, rs8192675-A, rs11924032-G, rs9875793-G) were found to be associated with higher susceptibility to the disease or its complications. The functions of ASC in the central nervous system and the brain have been extensively reviewed (110). Neurons have high concentrations of oxidative metabolism, 10-fold higher rates than supporting glia, which make them particularly vulnerable

to ASC deficiency (111, 112). The neuronal sensitivity to low supply of ASC is most apparent in neurodegenerative disease conditions in which there is excess oxidant stress and high oxidation rate of ASC to DHA (110). Radiotracer experiments have confirmed that DHA enters the brain and is converted to ASC (113). Therefore, in neurodegenerative disease such as bipolar disorder, DHA-GLUT transporters including SLC2A2, which is highly expressed in the brain, may play a key role to uptake the DHA and thus increase cerebral ASC concentrations to counter the oxidative stress resulting from the disease.

3.3.4.4 Liver disease

Genetic variants in *SLC2A1* are observed to actively contribute to nonalcoholic fatty liver disease (NAFLD), independent from diabetes or obesity (97). In a study on 520 patients with NAFLD and 521 healthy controls as well as 4,414 individuals with type 2 diabetes and 4,567 matched controls (97), genotypes rs4658-G/G and rs841856-T/T of *SLC2A1* showed association with increased risk of NAFLD, but not with diabetes. In this study gene-expression analysis demonstrated a considerable down-regulation of *SLC2A1* in liver of NAFLD patients. Moreover, in vitro silencing of *SLC2A1* resulted in increased oxidative stress and a higher lipid accumulation (97). SLC2A1 is involved in the DHA transport into mitochondria, resulting in mitochondrial vitamin C recycling and elevating protection against reactive oxygen species (97, 114). The mitochondrion has a key role in progression of nonalcoholic fatty liver disease (NAFLD) through impairing fatty liver homeostasis as well as inducing overproduction of reactive oxygen species and thus lipid peroxidation (97, 115). Variation in *SLC2A1* results in

mitochondrial redox imbalance hence could increase reactive oxygen species and regulate pro-inflammatory environment at early stages of the disease (97).

3.4. CONCLUSIONS AND FUTURE DIRECTIONS

Previously, observational studies have demonstrated that low vitamin C status increases the risk of many common chronic diseases. Today, genetic association studies on transporters of both Vitamin C transport pathways support and expand on these observational findings. This review stresses the importance to consider and investigate genetic variations impacting overall status, but also local tissue, and cell concentrations of vitamin C to sustain health and prevent common complex disease. As research progresses, it will be determined if human genetic variation on vitamin C transporters impact local or systemic pharmacokinetics. If pharmacokinetics is affected, recommendations will need to be adjusted for individuals or population subgroups of certain genotypes. This is apparent through the differential distributions of functional SNPs between African American and Caucasian individuals. Studies on variation in the genes coding different forms of vitamin C transporters are progressing and the evidence could be incorporated into future dietary guidelines. However, the emerging evidence, as previously proposed by others (6, 42, 116), needs further replications, biological proof, and dietary interventions studies in targeted individuals carrying the specific variants, in order to stand as valid diagnostic biomarkers. Moreover, the emerging fields of epigenetics and microbial analyses will contribute to the understanding of systematic interactions, and future studies will have to find a way to integrate genetics, epigenetics and metagenomics data.

3.5 REFERENCES

1. Levine, M. 1986. New concepts in the biology and biochemistry of ascorbic acid. *N Engl J Med* 314:892-902.

2. Frei, B., England, L., and Ames, B.N. 1989. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci U S A* 86:6377-6381.

3. Frei, B., Stocker, R., and Ames, B.N. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci U S A* 85:9748-9752.

4. Niki, E. 1987. Interaction of ascorbate and alpha-tocopherol. *Ann N Y Acad Sci* 498:186-199.

5. Packer, J.E., Slater, T.F., and Willson, R.L. 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 278:737-738.

6. Frei, B., Birlouez-Aragon, I., and Lykkesfeldt, J. 2012. Authors' perspective: What is the optimum intake of vitamin C in humans? *Crit Rev Food Sci Nutr* 52:815-829.

7. Agarwal, M., Mehta, P.K., Dwyer, J.H., Dwyer, K.M., Shircore, A.M., Nordstrom, C.K., Sun, P., Paul-Labrador, M., Yang, Y., and Merz, C.N. 2012. Differing Relations to Early Atherosclerosis between Vitamin C from Supplements vs. Food in the Los Angeles Atherosclerosis Study: A Prospective Cohort Study. *Open Cardiovasc Med J* 6:113-121.

8. Langlois, M., Duprez, D., Delanghe, J., De Buyzere, M., and Clement, D.L. 2001. Serum vitamin C concentration is low in peripheral arterial disease and is associated with inflammation and severity of atherosclerosis. *Circulation* 103:1863-1868.

9. Myint, P.K., Luben, R.N., Welch, A.A., Bingham, S.A., Wareham, N.J., and Khaw, K.T. 2008. Plasma vitamin C concentrations predict risk of incident stroke over 10 y in 20 649 participants of the European Prospective Investigation into Cancer Norfolk prospective population study. *Am J Clin Nutr* 87:64-69.

10. Simon, J.A., and Hudes, E.S. 1998. Serum ascorbic acid and other correlates of gallbladder disease among US adults. *Am J Public Health* 88:1208-1212.

11. Simon, J.A., Hudes, E.S., and Browner, W.S. 1998. Serum ascorbic acid and cardiovascular disease prevalence in U.S. adults. *Epidemiology* 9:316-321.

12. Tveden-Nyborg, P., and Lykkesfeldt, J. 2013. Does vitamin C deficiency increase lifestyle-associated vascular disease progression? Evidence based on experimental and clinical studies. *Antioxid Redox Signal* 19:2084-2104.

13. Schleicher, R.L., Carroll, M.D., Ford, E.S., and Lacher, D.A. 2009. Serum vitamin C and the prevalence of vitamin C deficiency in the United States: 2003-2004 National Health and Nutrition Examination Survey (NHANES). *Am J Clin Nutr* 90:1252-1263.

14. Siega-Riz, A.M., Promislow, J.H., Savitz, D.A., Thorp, J.M., Jr., and McDonald, T. 2003. Vitamin C intake and the risk of preterm delivery. *Am J Obstet Gynecol* 189:519-525.

15. Block, G. 1991. Vitamin C and cancer prevention: the epidemiologic evidence. *Am J Clin Nutr* 53:270S-282S.

16. Khaw, K.T., Bingham, S., Welch, A., Luben, R., Wareham, N., Oakes, S., and Day, N. 2001. Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. European Prospective Investigation into Cancer and Nutrition. *Lancet* 357:657-663.

17. Pfister, R., Sharp, S.J., Luben, R., Wareham, N.J., and Khaw, K.T. 2011. Plasma vitamin C predicts incident heart failure in men and women in European Prospective Investigation into Cancer and Nutrition-Norfolk prospective study. *Am Heart J* 162:246-253.

18. Jenab, M., Riboli, E., Ferrari, P., Sabate, J., Slimani, N., Norat, T., Friesen, M., Tjonneland, A., Olsen, A., Overvad, K., et al. 2006. Plasma and dietary vitamin C levels and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST). *Carcinogenesis* 27:2250-2257.

19. Simon, J.A., and Hudes, E.S. 1999. Serum ascorbic acid and cardiovascular disease prevalence in U.S. adults: the Third National Health and Nutrition Examination Survey (NHANES III). *Ann Epidemiol* 9:358-365.

20. Lindblad, M., Tveden-Nyborg, P., and Lykkesfeldt, J. 2013. Regulation of vitamin C homeostasis during deficiency. *Nutrients* 5:2860-2879.

21. Daruwala, R., Song, J., Koh, W.S., Rumsey, S.C., and Levine, M. 1999. Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. *FEBS Lett* 460:480-484.

22. Savini, I., Rossi, A., Pierro, C., Avigliano, L., and Catani, M.V. 2008. SVCT1 and SVCT2: key proteins for vitamin C uptake. *Amino Acids* 34:347-355.

23. Wilson, J.X. 2005. Regulation of vitamin C transport. *Annu Rev Nutr* 25:105-125.

24. Rumsey, S.C., Kwon, O., Xu, G.W., Burant, C.F., Simpson, I., and Levine, M. 1997. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem* 272:18982-18989.

25. Montel-Hagen, A., Kinet, S., Manel, N., Mongellaz, C., Prohaska, R., Battini, J.L., Delaunay, J., Sitbon, M., and Taylor, N. 2008. Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. *Cell* 132:1039-1048.

26. Rumsey, S.C., Daruwala, R., Al-Hasani, H., Zarnowski, M.J., Simpson, I.A., and Levine, M. 2000. Dehydroascorbic acid transport by GLUT4 in Xenopus oocytes and isolated rat adipocytes. *J Biol Chem* 275:28246-28253.

27. Corpe, C.P., Eck, P., Wang, J., Al-Hasani, H., and Levine, M. 2013. Intestinal dehydroascorbic acid (DHA) transport mediated by the facilitative sugar transporters, GLUT2 and GLUT8. *J Biol Chem* 288:9092-9101.

28. Lykkesfeldt, J. 2002. Increased oxidative damage in vitamin C deficiency is accompanied by induction of ascorbic acid recycling capacity in young but not mature guinea pigs. *Free Radic Res* 36:567-574.

29. Buettner, G.R. 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys* 300:535-543.

30. Linster, C.L., and Van Schaftingen, E. 2006. Glucuronate, the precursor of vitamin C, is directly formed from UDP-glucuronate in liver. *FEBS J* 273:1516-1527.

31. Corpe, C.P., Tu, H., Eck, P., Wang, J., Faulhaber-Walter, R., Schnermann, J., Margolis, S., Padayatty, S., Sun, H., Wang, Y., et al. 2010. Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice. *J Clin Invest* 120:1069-1083.

32. Sotiriou, S., Gispert, S., Cheng, J., Wang, Y., Chen, A., Hoogstraten-Miller, S., Miller, G.F., Kwon, O., Levine, M., Guttentag, S.H., et al. 2002. Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival. *Nature Medicine* 8:514-517.

33. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U.V., Chen, X.Z., Wang, Y., Brubaker, R.F., and Hediger, M.A. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 399:70-75.

34. Welch, R.W., Bergsten, P., Butler, J.D., and Levine, M. 1993. Ascorbic acid accumulation and transport in human fibroblasts. *Biochem J* 294 (Pt 2):505-510.

35. Godoy, A., Ormazabal, V., Moraga-Cid, G., Zuniga, F.A., Sotomayor, P., Barra, V., Vasquez, O., Montecinos, V., Mardones, L., Guzman, C., et al. 2007. Mechanistic insights and functional determinants of the transport cycle of the ascorbic acid transporter SVCT2. Activation by sodium and absolute dependence on bivalent cations. *J Biol Chem* 282:615-624.

36. Mackenzie, B., Illing, A.C., and Hediger, M.A. 2008. Transport model of the human Na+-coupled L-ascorbic acid (vitamin C) transporter SVCT1. *Am J Physiol Cell Physiol* 294:C451-459.

37. Eck, P., Erichsen, H.C., Taylor, J.G., Yeager, M., Hughes, A.L., Levine, M., and Chanock, S. 2004. Comparison of the genomic structure and variation in the two human sodium-dependent vitamin C transporters, SLC23A1 and SLC23A2. *Hum Genet* 115:285-294.

38. Wang, H., Dutta, B., Huang, W., Devoe, L.D., Leibach, F.H., Ganapathy, V., and Prasad, P.D. 1999. Human Na(+)-dependent vitamin C transporter 1 (hSVCT1): primary structure, functional characteristics and evidence for a non-functional splice variant. *Biochim Biophys Acta* 1461:1-9.

39. Wang, Y., Mackenzie, B., Tsukaguchi, H., Weremowicz, S., Morton, C.C., and Hediger, M.A. 2000. Human vitamin C (L-ascorbic acid) transporter SVCT1. *Biochem Biophys Res Commun* 267:488-494.

40. Amir Shaghaghi M, T.H., Yurkova N, Levine M, Eck P. 2013. Identification and Functional Characterization of an Alternative 5' Exon of the Sodium Dependent Ascorbic Acid Transporter SLC23A1. *J Hum Nutr Food Sci* 1: 1001.

41. InternationalHapMapConsortium. 2003. The International HapMap Project. *Nature* 426:789-796.

42. Michels, A.J., Hagen, T.M., and Frei, B. 2013. Human genetic variation influences vitamin C homeostasis by altering vitamin C transport and antioxidant enzyme function. *Annu Rev Nutr* 33:45-70.

43. Clark, A.G., Rohrbaugh, A.L., Otterness, I., and Kraus, V.B. 2002. The effects of ascorbic acid on cartilage metabolism in guinea pig articular cartilage explants. *Matrix Biol* 21:175-184.

44. Rajan, D.P., Huang, W., Dutta, B., Devoe, L.D., Leibach, F.H., Ganapathy, V., and Prasad, P.D. 1999. Human placental sodium-dependent vitamin C transporter (SVCT2): molecular cloning and transport function. *Biochem Biophys Res Commun* 262:762-768.

45. Liang, W.J., Johnson, D., and Jarvis, S.M. 2001. Vitamin C transport systems of mammalian cells. *Mol Membr Biol* 18:87-95.

46. Boyer, J.C., Campbell, C.E., Sigurdson, W.J., and Kuo, S.M. 2005. Polarized localization of vitamin C transporters, SVCT1 and SVCT2, in epithelial cells. *Biochem Biophys Res Commun* 334:150-156.

47. Varma, S., Sobey, K., Campbell, C.E., and Kuo, S.M. 2009. Hierarchal contribution of N- and C-terminal sequences to the differential localization of homologous sodium-

dependent vitamin C transporters, SVCT1 and SVCT2, in epithelial cells. *Biochemistry* 48:2969-2980.

48. Eck, P., Erichsen, H.C., Taylor, J.G., Corpe, C., Chanock, S.J., and Levine, M. 2007. Genomic and functional analysis of the sodium-dependent vitamin C transporter SLC23A1-SVCT1. *Genes Nutr* 2:143-145.

49. Skibola, C.F., Bracci, P.M., Halperin, E., Nieters, A., Hubbard, A., Paynter, R.A., Skibola, D.R., Agana, L., Becker, N., Tressler, P., et al. 2008. Polymorphisms in the estrogen receptor 1 and vitamin C and matrix metalloproteinase gene families are associated with susceptibility to lymphoma. *PLoS One* 3:e2816.

50. Erichsen, H.C., Peters, U., Eck, P., Welch, R., Schoen, R.E., Yeager, M., Levine, M., Hayes, R.B., and Chanock, S. 2008. Genetic variation in sodium-dependent vitamin C transporters SLC23A1 and SLC23A2 and risk of advanced colorectal adenoma. *Nutr Cancer* 60:652-659.

51. Chen, A.A., Marsit, C.J., Christensen, B.C., Houseman, E.A., McClean, M.D., Smith, J.F., Bryan, J.T., Posner, M.R., Nelson, H.H., and Kelsey, K.T. 2009. Genetic variation in the vitamin C transporter, SLC23A2, modifies the risk of HPV16-associated head and neck cancer. *Carcinogenesis* 30:977-981.

52. Minegaki, T., Kuwahara, A., Yamamori, M., Nakamura, T., Okuno, T., Miki, I., Omatsu, H., Tamura, T., Hirai, M., Azuma, T., et al. 2014. Genetic polymorphisms in SLC23A2 as predictive biomarkers of severe acute toxicities after treatment with a definitive 5-fluorouracil/cisplatin-based chemoradiotherapy in Japanese patients with esophageal squamous cell carcinoma. *Int J Med Sci* 11:321-326.

53. Wright, M.E., Andreotti, G., Lissowska, J., Yeager, M., Zatonski, W., Chanock, S.J., Chow, W.H., and Hou, L. 2009. Genetic variation in sodium-dependent ascorbic acid transporters and risk of gastric cancer in Poland. *Eur J Cancer* 45:1824-1830.

54. Duell, E.J., Lujan-Barroso, L., Llivina, C., Munoz, X., Jenab, M., Boutron-Ruault, M.C., Clavel-Chapelon, F., Racine, A., Boeing, H., Buijsse, B., et al. 2013. Vitamin C transporter gene (SLC23A1 and SLC23A2) polymorphisms, plasma vitamin C levels, and gastric cancer risk in the EPIC cohort. *Genes Nutr* 8:549-560.

55. Andrew, A.S., Gui, J., Sanderson, A.C., Mason, R.A., Morlock, E.V., Schned, A.R., Kelsey, K.T., Marsit, C.J., Moore, J.H., and Karagas, M.R. 2009. Bladder cancer SNP panel predicts susceptibility and survival. *Hum Genet* 125:527-539.

56. Amir Shaghaghi, M., Bernstein, Charles N., Serrano Leo´n, Alejandra., El-Gabalawy, Hani., Eck, Peter. 2014. Polymorphisms in the sodium-dependent ascorbate transporter gene SLC23A1 are associated with susceptibility to Crohn disease. *Am J Clin Nutr* 99:378–383.

57. Erichsen, H.C., Engel, S.A., Eck, P.K., Welch, R., Yeager, M., Levine, M., Siega-Riz, A.M., Olshan, A.F., and Chanock, S.J. 2006. Genetic variation in the sodium-dependent vitamin C transporters, SLC23A1, and SLC23A2 and risk for preterm delivery. *Am J Epidemiol* 163:245-254.

58. Dalgard, C., Christiansen, L., Vogel, U., Dethlefsen, C., Tjonneland, A., and Overvad, K. 2013. Variation in the sodium-dependent vitamin C transporter 2 gene is associated with risk of acute coronary syndrome among women. *PLoS One* 8:e70421.

59. Zanon-Moreno, V., Ciancotti-Olivares, L., Asencio, J., Sanz, P., Ortega-Azorin, C., Pinazo-Duran, M.D., and Corella, D. 2011. Association between a SLC23A2 gene variation, plasma vitamin C levels, and risk of glaucoma in a Mediterranean population. *Mol Vis* 17:2997-3004.

60. Senthilkumari, S., Talwar, B., Dharmalingam, K., Ravindran, R.D., Jayanthi, R., Sundaresan, P., Saravanan, C., Young, I.S., Dangour, A.D., and Fletcher, A.E. 2014. Polymorphisms in sodium-dependent vitamin C transporter genes and plasma, aqueous humor and lens nucleus ascorbate concentrations in an ascorbate depleted setting. *Exp Eye Res* 124:24-30.

61. Lee, S.K., Kang, J.S., Jung da, J., Hur, D.Y., Kim, J.E., Hahm, E., Bae, S., Kim, H.W., Kim, D., Cho, B.J., et al. 2008. Vitamin C suppresses proliferation of the human melanoma cell SK-MEL-2 through the inhibition of cyclooxygenase-2 (COX-2) expression and the modulation of insulin-like growth factor II (IGF-II) production. *J Cell Physiol* 216:180-188.

62. Cui, Y., Lu, Z., Bai, L., Shi, Z., Zhao, W.E., and Zhao, B. 2007. beta-Carotene induces apoptosis and up-regulates peroxisome proliferator-activated receptor gamma expression and reactive oxygen species production in MCF-7 cancer cells. *Eur J Cancer* 43:2590-2601.

63. Yu, W., Sanders, B.G., and Kline, K. 2003. RRR-alpha-tocopheryl succinate-induced apoptosis of human breast cancer cells involves Bax translocation to mitochondria. *Cancer Res* 63:2483-2491.

64. Seifried, H.E., Anderson, D.E., Sorkin, B.C., and Costello, R.B. 2004. Free radicals: the pros and cons of antioxidants. Executive summary report. *J Nutr* 134:3143S-3163S.

65. Drake, I.M., Davies, M.J., Mapstone, N.P., Dixon, M.F., Schorah, C.J., White, K.L., Chalmers, D.M., and Axon, A.T. 1996. Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. *Carcinogenesis* 17:559-562.

66. Sasazuki, S., Hayashi, T., Nakachi, K., Sasaki, S., Tsubono, Y., Okubo, S., Hayashi, M., and Tsugane, S. 2008. Protective effect of vitamin C on oxidative stress: a randomized controlled trial. *Int J Vitam Nutr Res* 78:121-128.

67. Catani, M.V., Savini, I., Rossi, A., Melino, G., and Avigliano, L. 2005. Biological role of vitamin C in keratinocytes. *Nutr Rev* 63:81-90.

68. Kruidenier, L., and Verspaget, H.W. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 16:1997-2015.

69. Wideman, G.L., Baird, G.H., and Bolding, O.T. 1964. Ascorbic Acid Deficiency and Premature Rupture of Fetal Membranes. *Am J Obstet Gynecol* 88:592-595.

70. Guajardo, L., Beharry, K.D., Modanlou, H.D., and Aranda, J.V. 1995. Ascorbic acid concentrations in umbilical cord veins and arteries of preterm and term newborns. *Biol Neonate* 68:1-9.

71. Steyn, P.S., Odendaal, H.J., Schoeman, J., Stander, C., Fanie, N., and Grove, D. 2003. A randomised, double-blind placebo-controlled trial of ascorbic acid supplementation for the prevention of preterm labour. *J Obstet Gynaecol* 23:150-155.

72. Barrett, B.M., Sowell, A., Gunter, E., and Wang, M. 1994. Potential role of ascorbic acid and beta-carotene in the prevention of preterm rupture of fetal membranes. *Int J Vitam Nutr Res* 64:192-197.

73. Casanueva, E., Magana, L., Pfeffer, F., and Baez, A. 1991. Incidence of premature rupture of membranes in pregnant women with low leukocyte levels of vitamin C. *Eur J Clin Nutr* 45:401-405.

74. Nakata, Y., and Maeda, N. 2002. Vulnerable atherosclerotic plaque morphology in apolipoprotein E-deficient mice unable to make ascorbic Acid. *Circulation* 105:1485-1490.

75. Izzotti, A., Bagnis, A., and Sacca, S.C. 2006. The role of oxidative stress in glaucoma. *Mutat Res* 612:105-114.

76. Kang, J.H., Pasquale, L.R., Willett, W., Rosner, B., Egan, K.M., Faberowski, N., and Hankinson, S.E. 2003. Antioxidant intake and primary open-angle glaucoma: a prospective study. *Am J Epidemiol* 158:337-346.

77. Yuki, K., Murat, D., Kimura, I., Ohtake, Y., and Tsubota, K. 2010. Reduced-serum vitamin C and increased uric acid levels in normal-tension glaucoma. *Graefes Arch Clin Exp Ophthalmol* 248:243-248.

78. Leite, M.T., Prata, T.S., Kera, C.Z., Miranda, D.V., de Moraes Barros, S.B., and Melo, L.A., Jr. 2009. Ascorbic acid concentration is reduced in the secondary aqueous humour of glaucomatous patients. *Clin Experiment Ophthalmol* 37:402-406.

79. Schorah, C.J. 1992. The transport of vitamin C and effects of disease. *Proc Nutr Soc* 51:189-198.

80. Daskalopoulos, R., Korcok, J., Tao, L., and Wilson, J.X. 2002. Accumulation of intracellular ascorbate from dehydroascorbic acid by astrocytes is decreased after oxidative stress and restored by propofol. *Glia* 39:124-132.

81. Himmelreich, U., Drew, K.N., Serianni, A.S., and Kuchel, P.W. 1998. 13C NMR studies of vitamin C transport and its redox cycling in human erythrocytes. *Biochemistry* 37:7578-7588.

82. Holmes, M.E., Mwanjewe, J., Samson, S.E., Haist, J.V., Wilson, J.X., Dixon, S.J., Karmazyn, M., and Grover, A.K. 2002. Dehydroascorbic acid uptake by coronary artery smooth muscle: effect of intracellular acidification. *Biochem J* 362:507-512.

83. Kodaman, P.H., and Behrman, H.R. 1999. Hormone-regulated and glucose-sensitive transport of dehydroascorbic acid in immature rat granulosa cells. *Endocrinology* 140:3659-3665.

84. Mendiratta, S., Qu, Z.C., and May, J.M. 1998. Erythrocyte ascorbate recycling: antioxidant effects in blood. *Free Radic Biol Med* 24:789-797.

85. Qutob, S., Dixon, S.J., and Wilson, J.X. 1998. Insulin stimulates vitamin C recycling and ascorbate accumulation in osteoblastic cells. *Endocrinology* 139:51-56.

86. Upston, J.M., Karjalainen, A., Bygrave, F.L., and Stocker, R. 1999. Efflux of hepatic ascorbate: a potential contributor to the maintenance of plasma vitamin C. *Biochem J* 342 (Pt 1):49-56.

87. Malo, C., and Wilson, J.X. 2000. Glucose modulates vitamin C transport in adult human small intestinal brush border membrane vesicles. *J Nutr* 130:63-69.

88. Vera, J.C., Rivas, C.I., Velasquez, F.V., Zhang, R.H., Concha, II, and Golde, D.W. 1995. Resolution of the facilitated transport of dehydroascorbic acid from its intracellular accumulation as ascorbic acid. *J Biol Chem* 270:23706-23712.

89. Vera, J.C., Rivas, C.I., Fischbarg, J., and Golde, D.W. 1993. Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. *Nature* 364:79-82.

90. Mardones, L., Ormazabal, V., Romo, X., Jana, C., Binder, P., Pena, E., Vergara, M., and Zuniga, F.A. 2011. The glucose transporter-2 (GLUT2) is a low affinity dehydroascorbic acid transporter. *Biochem Biophys Res Commun* 410:7-12.

91. Zhao, F.Q., and Keating, A.F. 2007. Functional properties and genomics of glucose transporters. *Curr Genomics* 8:113-128.

92. Zhao, F.Q., Glimm, D.R., and Kennelly, J.J. 1993. Distribution of mammalian facilitative glucose transporter messenger RNA in bovine tissues. *Int J Biochem* 25:1897-1903.

93. Kellett, G.L., and Brot-Laroche, E. 2005. Apical GLUT2: a major pathway of intestinal sugar absorption. *Diabetes* 54:3056-3062.

94. Hsu, C.C., Kao, W.L., Steffes, M.W., Gambir, T., Brancati, F.L., Heilig, C.W., Shuldiner, A.R., Boerwinkle, E.A., and Coresh, J. 2011. Genetic variation of glucose transporter-1 (GLUT1) and albuminuria in 10,278 European Americans and African Americans: a case-control study in the Atherosclerosis Risk in Communities (ARIC) study. *BMC Med Genet* 12:16.

95. Roy, M.S., Hallman, D.M., Fu, Y.P., Machado, M., and Hanis, C.L. 2009. Assessment of 193 candidate genes for retinopathy in African Americans with type 1 diabetes. *Arch Ophthalmol* 127:605-612.

96. Page, T., Hodgkinson, A.D., Ollerenshaw, M., Hammonds, J.C., and Demaine, A.G. 2005. Glucose transporter polymorphisms are associated with clear-cell renal carcinoma. *Cancer Genet Cytogenet* 163:151-155.

97. Vazquez-Chantada, M., Gonzalez-Lahera, A., Martinez-Arranz, I., Garcia-Monzon, C., Regueiro, M.M., Garcia-Rodriguez, J.L., Schlangen, K.A., Mendibil, I., Rodriguez-Ezpeleta, N., Lozano, J.J., et al. 2013. Solute carrier family 2 member 1 is involved in the development of nonalcoholic fatty liver disease. *Hepatology* 57:505-514.

98. Davidson, C.M., Northrup, H., King, T.M., Fletcher, J.M., Townsend, I., Tyerman, G.H., and Au, K.S. 2008. Genes in glucose metabolism and association with spina bifida. *Reprod Sci* 15:51-58.

99. Laukkanen, O., Lindstrom, J., Eriksson, J., Valle, T.T., Hamalainen, H., Ilanne-Parikka, P., Keinanen-Kiukaanniemi, S., Tuomilehto, J., Uusitupa, M., Laakso, M., et al. 2005. Polymorphisms in the SLC2A2 (GLUT2) gene are associated with the conversion from impaired glucose tolerance to type 2 diabetes: the Finnish Diabetes Prevention Study. *Diabetes* 54:2256-2260.

100. Willer, C.J., Bonnycastle, L.L., Conneely, K.N., Duren, W.L., Jackson, A.U., Scott, L.J., Narisu, N., Chines, P.S., Skol, A., Stringham, H.M., et al. 2007. Screening of 134 single nucleotide polymorphisms (SNPs) previously associated with type 2 diabetes replicates association with 12 SNPs in nine genes. *Diabetes* 56:256-264.

101. Meyer, T.E., Boerwinkle, E., Morrison, A.C., Volcik, K.A., Sanderson, M., Coker, A.L., Pankow, J.S., and Folsom, A.R. 2010. Diabetes genes and prostate cancer in the Atherosclerosis Risk in Communities study. *Cancer Epidemiol Biomarkers Prev* 19:558-565.

102. Dupuis, J., Langenberg, C., Prokopenko, I., Saxena, R., Soranzo, N., Jackson, A.U., Wheeler, E., Glazer, N.L., Bouatia-Naji, N., Gloyn, A.L., et al. 2010. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. *Nat Genet* 42:105-116.

103. Borglykke, A., Grarup, N., Sparso, T., Linneberg, A., Fenger, M., Jeppesen, J., Hansen, T., Pedersen, O., and Jorgensen, T. 2012. Genetic variant SLC2A2 [corrected] Is associated with risk of cardiovascular disease - assessing the individual and cumulative effect of 46 type 2 diabetes related genetic variants. *PLoS One* 7:e50418.

104. Meier, S., Mattheisen, M., Vassos, E., Strohmaier, J., Treutlein, J., Josef, F., Breuer, R., Degenhardt, F., Muhleisen, T.W., Muller-Myhsok, B., et al. 2012. Genome-wide significant association between a 'negative mood delusions' dimension in bipolar disorder and genetic variation on chromosome 3q26.1. *Transl Psychiatry* 2:e165.

105. Le, M.T., Lobmeyer, M.T., Campbell, M., Cheng, J., Wang, Z., Turner, S.T., Chapman, A.B., Boerwinkle, E., Gums, J.G., Gong, Y., et al. 2013. Impact of genetic polymorphisms of SLC2A2, SLC2A5, and KHK on metabolic phenotypes in hypertensive individuals. *PLoS One* 8:e52062.

106. Yin, T., Li, N.F., Heizhati, M., Zhang, J., Zhang, J., Zhou, L., and Chang, G. 2014. Association of glucose transporter 4 genetic polymorphisms with obstructive sleep apnea syndrome in Han Chinese general population: a cross-section study. *Lipids Health Dis* 13:12.

107. Abhary, S., Hewitt, A.W., Burdon, K.P., and Craig, J.E. 2009. A systematic meta-analysis of genetic association studies for diabetic retinopathy. *Diabetes* 58:2137-2147.

108. Hodgkinson, A.D., Millward, B.A., and Demaine, A.G. 2001. Polymorphisms of the glucose transporter (GLUT1) gene are associated with diabetic nephropathy. *Kidney Int* 59:985-989.

109. Campa, D., Husing, A., Chang-Claude, J., Dostal, L., Boeing, H., Kroger, J., Tjonneland, A., Roswall, N., Overvad, K., Dahm, C.C., et al. 2011. Genetic variability of the fatty acid synthase pathway is not associated with prostate cancer risk in the European Prospective Investigation on Cancer (EPIC). *Eur J Cancer* 47:420-427.

110. Harrison, F.E., and May, J.M. 2009. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. *Free Radic Biol Med* 46:719-730.

111. Wilson, J.X. 1997. Antioxidant defense of the brain: a role for astrocytes. *Can J Physiol Pharmacol* 75:1149-1163.

112. Hediger, M.A. 2002. New view at C. *Nat Med* 8:445-446.

113. Agus, D.B., Gambhir, S.S., Pardridge, W.M., Spielholz, C., Baselga, J., Vera, J.C., and Golde, D.W. 1997. Vitamin C crosses the blood-brain barrier in the oxidized form through the glucose transporters. *J Clin Invest* 100:2842-2848.

114. Kc, S., Carcamo, J.M., and Golde, D.W. 2005. Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury. *FASEB J* 19:1657-1667.

115. Serviddio, G., Bellanti, F., Vendemiale, G., and Altomare, E. 2011. Mitochondrial dysfunction in nonalcoholic steatohepatitis. *Expert Rev Gastroenterol Hepatol* 5:233-244.

116. Lykkesfeldt, J., and Poulsen, H.E. 2010. Is vitamin C supplementation beneficial? Lessons learned from randomised controlled trials. *Br J Nutr* 103:1251-1259.

TRANSITION STATEMENT 1

As one of the main objective of this thesis being to investigate the genetic structure and disease association of intestinal vitamin C transporter SLC23A1, we decided to revisit the structure of its responsible gene in order to identify its splice variants and to further investigate the functional properties of each splice variant.

Previous animal studies showed that *Slc23a1* is a key gene/protein for intestinal absorption, hepatic handling and renal re-absorption of ascorbic acid. However, the human *SLC23A1* is somewhat understudied, and the function of SLC23A1 as a transporter, makes the gene a candidate for alternative splicing. Through *in silico* analysis, we found promising evidence supporting the existence of an alternative splice variant utilizing an alternative first exon. Alternative splicing results in diverse proteins with different or even antagonistic functional and structural properties, from the same protein coding gene. As such, to validate any possible disease association of a gene a complete annotation of the gene locus and function is required.

In the following manuscript, we verified the *In silco* evidence experimentally, and report the identification, and functional characterizations of a novel alternative first exon. This is the first report of an alternative exon in the human *SLC23A1* gene since the initial description in 1999/2000.

CHAPTER 4

MANUSCRIPT 2

This manuscript was published in the Journal of Human Nutrition & Food Science

(*J Hum Nutr Food Sci 1: 1001.*

The manuscript was chosen as "The feature Article of the Month" for the journal (August 2013)

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF AN ALTERNATIVE 5' EXON OF THE SODIUM DEPENDENT ASCORBIC ACID TRANSPORTER SLC23A1

Mandana Amir Shaghaghi[1], Hongbin Tu[2], Natalia Yurkova[1], Mark Levine[2], Peter Eck[1]

Afflications: [1]University of Manitoba, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada. [2]Molecular and Clinical Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

4.1 ABSTRACT

Since the initial description of the human *SLC23A1* transcript, encoding a sodium-dependent ascorbic acid transporter, no alternative splice variant had been described. In this report we describe a novel human specific alternative first exon which is located 1078 nucleotides 5' of the canonical first exon. The two first exons are not mutually exclusive since they splice together to create a novel SLC23A1 protein isoform, we name it isoform 1A, with a N-terminus that is elongated by 36 amino acid. The novel SLC23A1 isoform locates to the plasma membrane, but mediates only 7% of the ascorbic acid transport exhibited by the shorter isoform when expressed in Xenopus laevis oocytes.

4.2 INTRODUCTION

The *SLC23A1* locus on human chromosome 5q31.2 (138702885-138719039 compl.; NC_000005.9) encodes for the sodium-dependent ascorbic acid transporter 1. Ascorbic acid, the bioactive form of Vitamin C is essential for human survival and the prevention of common complex diseases (1, 2). Main sites of SLC23A1 expression are the small intestine, liver and kidney, where it functions as an apical ascorbic acid uptake carrier (3-9). SLC23A1 transports ascorbic acid with moderate affinity (K_m ~ 100-200 µM) and high capacity, reflecting its role in intestinal absorption and renal re-absorption (3, 4, 10). Two alternative splice variants have been described in the literature and are listed in the NCBI database as reference RNAs NM005847.4 and NM152685.3. The protein isoform represented by the splice variant NM152685.3 mediates cellular ascorbic acid uptake (5, 11, 12), while the isoform encoded by NM005847.4, which

contains additional 12 nucleotides between exon 5 and 6 adding 4 amino acids, shows no ASC transport (5).

The *SLC23A1* exon intron structure has been described as containing 15 exons, stretching over 16 kilobases (13, 14), and is similar to the mouse ortholog (15). SLC23A1 is the key protein in renal re-absorption and hepatic maintenance of ascorbic acid (16), making it a major determinant of systemic ascorbic acid levels. Genetic variation in SLC23A1 has a high potential to impact on systemic ascorbic acid levels, and the potential detrimental health impact is demonstrated by the fact that the *slc23a1*$^{-/-}$ mouse has low plasma and tissue concentrations and ~50% perinatal mortality (16).

Most genes in the human genome have more than two splice variants(17, 18); therefore we evaluated the existence of additional SLC23A1 transcripts *in silico.* In this report we characterize a novel alternative first exon encoding a SLC23A1 isoform which is elongated by 36 additional N-terminal amino acids on a genomic and functional level.

4.3 MATERIAL AND METHODS

4.3.1 EXPRESSED SEQUENCE TAGS (EST) SEARCH AND ALIGNMENTS

All mRNA and EST sequences in the human SLC23A1 5' region were identified by BLASTing the known SLC23A1 Exon (1B) and intron 1, as well as the 5000 nucleotides in the proximal promoter region (NCBI Human Genome Build 37.3) against the human Non-RefSeq RNA and EST databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Data were downloaded, assembled and

visually analyzed (curated) using Sequencher 4.9, changing alignment parameter as needed. Data were curated and validated visually and compared to data available from the NCBI and Ensembl databases for verification. Evidence for expression in non humanoid life forms was evaluated through BLAST searches in databases for all available species (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.2 COMPARATIVE CONSERVATION ANALYSIS USING VISTA TOOLS

Using the human genome (NCBI Human Genome Build 37.3) as the base genome, candidate orthologous regions were identified using gVISTA (http://genome.lbl.gov/cgi-bin/GenomeVista). The sequence of the novel *SLC23A1* Exon1A was blasted in gVISTA (http://genome.lbl.gov/cgi-bin/GenomeVista) to pinpoint its precise genomic location. Conservation in the latest pre-computed genomic assemblies were compared in the VISTA browser (http://pipeline.lbl.gov/cgi-bin/gateway2?selector=vistapoint).

4.3.3 REAL TIME PCR

Real Time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) following manufactures recommendations. StepOnePlus operation was controlled by the StepOne software vs. 2.2.2, which also used for data processing and analysis. Final analysis was confirmed using Microsoft Excel, which was also used to design graphics.

PrimeTime® TaqMan based qPCR assay IDT47803527 (Integrated DNA Technologies, Coralville, IA, USA) was used to determine levels of the canonical consensus *SLC23A1* transcript represented by NM005847.4 and NM152685.3. PrimeTime® TaqMan based qPCR assay IDT52386812 (Integrated DNA Technologies, Coralville, IA, USA) was used to determine levels of the transcript containing the novel *SLC23A1* exon 1A. Assays were performed in TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA, USA) following the manufacturers protocols. Clontech human liver, small intestine and kidney Marathon ready cDNA (Clontech, Mountain View, CA, USA), which had been standardized based on β–actin levels by the manufacturer, was used in the qPCR. Plasmids representing the *SLC23A1* consensus as well as exon 1A transcript were used in serial dilutions to determine a standard curve, where plasmids copy numbers were calculated based on the formula: m=(n)x1.096e-21g (m=mass, n=plasmid size in nucleotides).

4.3.4 CLONING OF THE *SLC23A1* EXON1A CDNA

For all PCR based subcloning steps Phusion High-Fidelity DNA Polymerase (New England BioLabs, Whitby, ON, Canada) was used according to the manufacturers recommendations. The identity of PCR amplicons and the final inserts in the DONR and expression plasmids had been confirmed by sequencing (The Centre for Applied Genomics, Toronto, ON, Canada) and sequence data were analyzed by Sequencher 5.0 (GeneCodes, Ann Arbor, MI, USA). A two step PCR methodology was applied to create the fluorescently tagged protein, where first two separate PCR products for the *SLC23A1* exon 1A ORF (template RNA was obtained from CaCo-2 cells) and turbo Green Fluorescent Protein (tGFP) (template GIPZ Lentiviral shRNAmir

vector, Open Biosystems) were produced (Table of primers in Supplement). These amplicons were designed to have overlapping sequences and in a second round of PCR these were used to obtain one amplicon, which was tagged with the Gateway™ (Invitrogen, Burlington, ON, Canada) recombination tags. This amplicons was integrated into pDONR22.1 and transferred into pcDNA/V5-DEST using the manufacturer's protocol. The ORF representing the shorter *SLC23A1* splice variant (NM005847.4) was contained in the m-cherry red fluorescent protein vector *mCherry-SLC23A1* pReceiver-M55 (GeneCopoeia, Rockville, MD, USA).

4.3.5 CELL CULTURE AND TRANSFECTION

HEK293 cells (ATCC) were cultured at 37°C in DMEM/F-12 HyClone media (Thermo Fisher Scientific, Burlington, ON, Canada), 10% fetal bovine serum (FBS) on 75mm flasks (Thermo Fisher Scientific, Corning, Burlington, ON, Canada)) or 24-well tissue culture plates (Thermo Fisher Scientific, BioLite, Burlington, ON, Canada)). At appropriate densities the cells were transfected with *mCherry-SLC23A1* pReceiver-M55 or *tGFP-SLC23A1 Exon1A pcDNA/V5-DEST* using the Effectene Transfection Reagent (Qiagen, Toronto, ON, Canada) following the manufacturer's protocols. 24 to 48h post transfection the cells were visualized by fluorescent microscopy (Axiovert 200, Zeiss, North York, ON, Canada).

4.3.6 FLUORESCENT MICROSCOPY

24 to 48h post transfection the fluorescence in the transfected cells was visualized using an Axiovert 200M inverted wide-field epifluorescence microscopy (Carl Zeiss, North York, ON, Canada). Images were collected with the Axiovision software package AxioVS40 v 4.5.

4.3.7 PROTEIN PREDICTION ANALYSIS

ProtParam (http://web.expasy.org/cgi-bin/protparam/protparam) was used for computation of various physical and chemical parameters, including the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

The TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the transmembrane architecture of the proteins. PredictProtein (https://www.predictprotein.org) was used to predict secondary structure, solvent accessibility, transmembrane helices, globular regions, coiled-coil regions, structural switch regions, B-values, disorder regions, intra-residue contacts, protein-protein and protein-DNA binding sites, sub-cellular localization, domain boundaries, beta-barrels, cysteine bonds, metal binding sites and disulphide bridges of the two proteins.

4.3.8 *XENOPUS LAEVIS* OOCYTES TRANSPORT ASSAY

Complementary RNAs (cRNA) for both human *SLC23A1* splice variants were prepared by *in*

vitro transcription using the SP6 mMessage mMachine Kit (Ambion, Burlington, ON, Canada). The cRNAs were injected into Xenopus laevis oocytes and the transporter experiments were performed exactly as described (16).

4.4 RESULTS

4.4.1 A NOVEL ALTERNATIVE FIRST *SLC23A1* EXON EXCLUSIVE TO HUMANS IS EXPRESSED IN THE SMALL INTESTINE

The existence of an alternative first *SLC23A1* exon is supported by five Expressed Sequence Tags (ESTs: DA420756, DB480870, DA870503, EG327910, EG328033) aligning 1078 nucleotides 5' of the currently known *SLC23A1* exon 1 **(Figure 4.1A).** These five ESTs define a novel first exon for the human *SLC23A1* gene, which we name *SLC23A1* exon 1A **(Figure 4.1A)**. The novel *SLC23A1* exon1A consists of 196 nucleotides, with the transcript splicing into the following exon sixty-two nucleotides from its 3' end **(Figure 4.1B).** The full length of the exon was confirmed by PCR amplification using RNA from the intestinal carcinoma cell line CaCo-2 as template and subsequent sequence analysis.

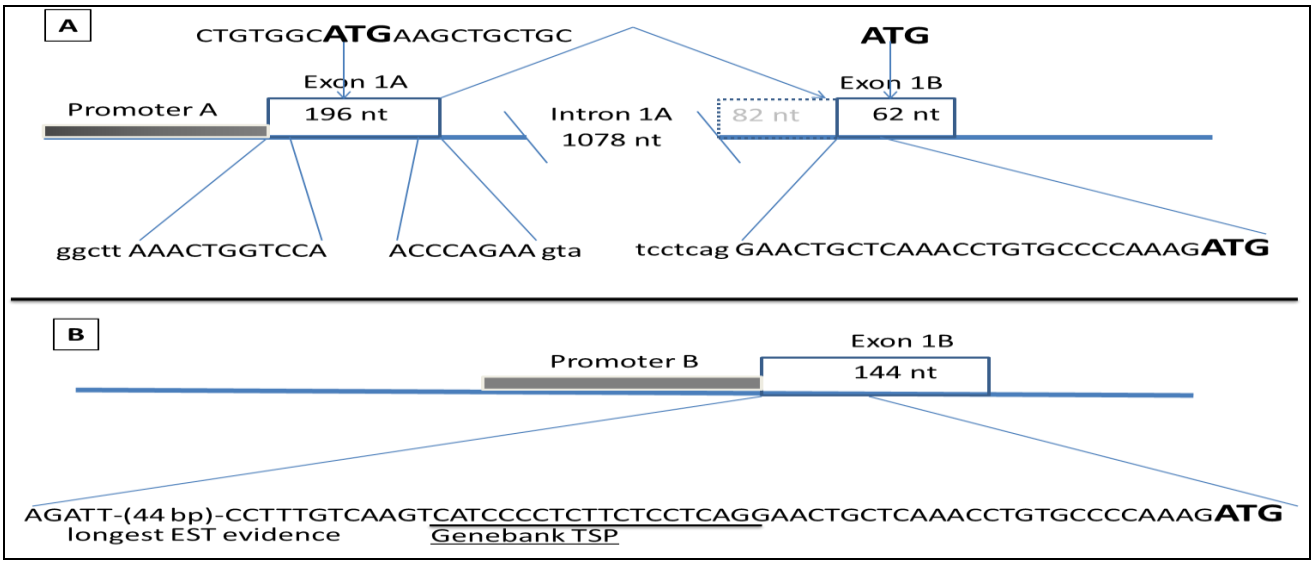


Figure 4.1: Exon usage in the 5' region of the SLC23A1 gene. **A)** Two alternative first exons are utilized. Exon 1A is the novel exon described in this report. Both first exons have independent promoter regions, but are not mutually exclusive, since exon 1A splices into exon 1B, which is the exon described before (13, 14) and defined by the reference RNAs NM005847.4 and NM152685.3. **B)** The Transcriptional Start Point (TSP) of SLC23A1 exon 1B is not represented by the reference RNAs, instead it extends 61 nucleotides 5', which brings the size of exon 1B to 144 nucleotides.

The novel *SLC23A1* exon1A is not reflected through any reference RNA in GeneBank. The reference RNAs NM005847.4 and NM152685.3 define the currently documented first exon (13, 14), which by virtue of chromosomal order is defined as *SLC23A1* Exon 1B **(Figure 4.1B)**.

The expression of the novel *SLC23A1* exon 1A is limited to humans, no transcript with a sequence homology of 70% or higher could be identified in any RNA or EST database. An analysis of the genomic region surrounding SLC23A1 Exon 1A shows conservation in primates, moderate conservation between human and equine (horse), canine (dog), and bovine (cow), and very low conservation between primates and rodents (mouse and rat) **(Figure 4.2)**. The low

evolutionary conservation, specifically in the proximal promoter region, supports the evidence that this splice variant appears to be unique to humans or primates.

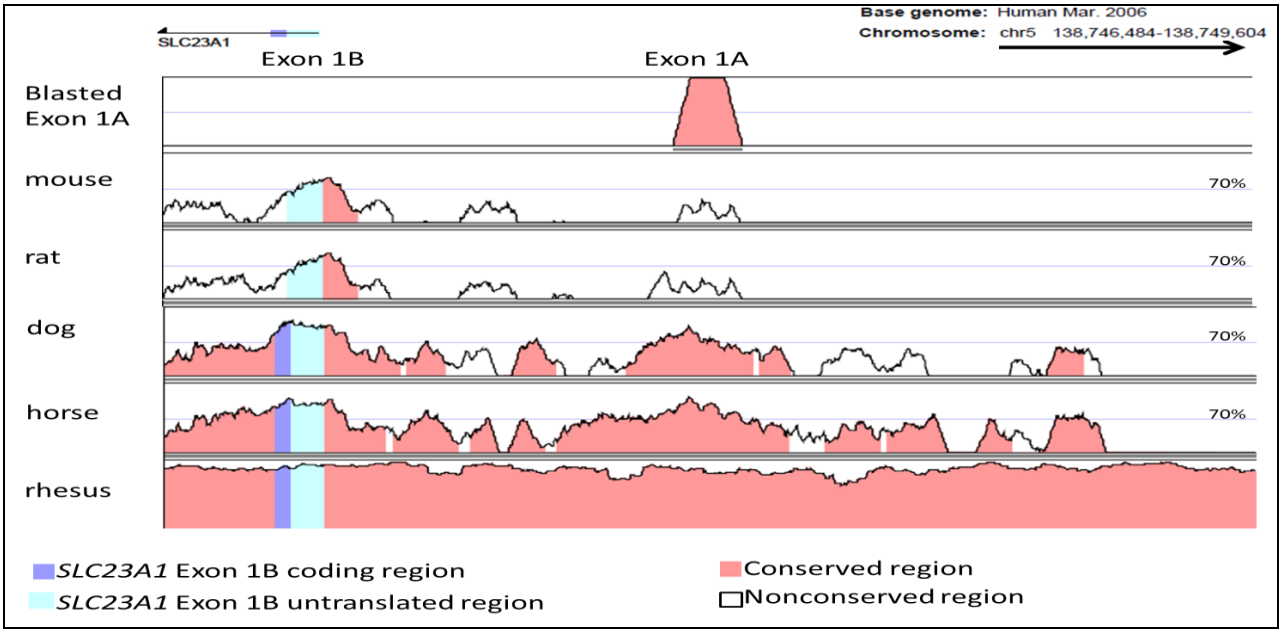


Figure 4.2: **Comparative VISTA Analysis of the evolutionary conservation of the orthologous regions containing the novel SLC23A1 Exon 1A and the previously characterized SLC23A1 Exon 1B.** SLC23A1 is located on the complement strand of chromosome 5. The first lane shows the exact location of the novel SLC23A1 Exon 1A, as determined in a gVISTA blast search. The threshold for high conservation was set to be 70%. SLC23A1 Exon 1A is not conserved in rodents, and moderately conserved in dog and cow, while SLC23A1 Exon 1B is highly conserved across all species.

Real time PCR analysis shows abundant expression of the full length *SLC23A1* transcript in the kidney, liver and small intestine **(Figure 4.3A),** as previously observed (5, 11, 12, 16, 19). In contrast, transcripts containing *SLC23A1* exon 1A are only detectable in trace amounts in liver and kidney **(Figure 4.3B)**, but constitute 18% of the *SLC23A1* transcripts in the small intestine **(Figure 4.3C)**.

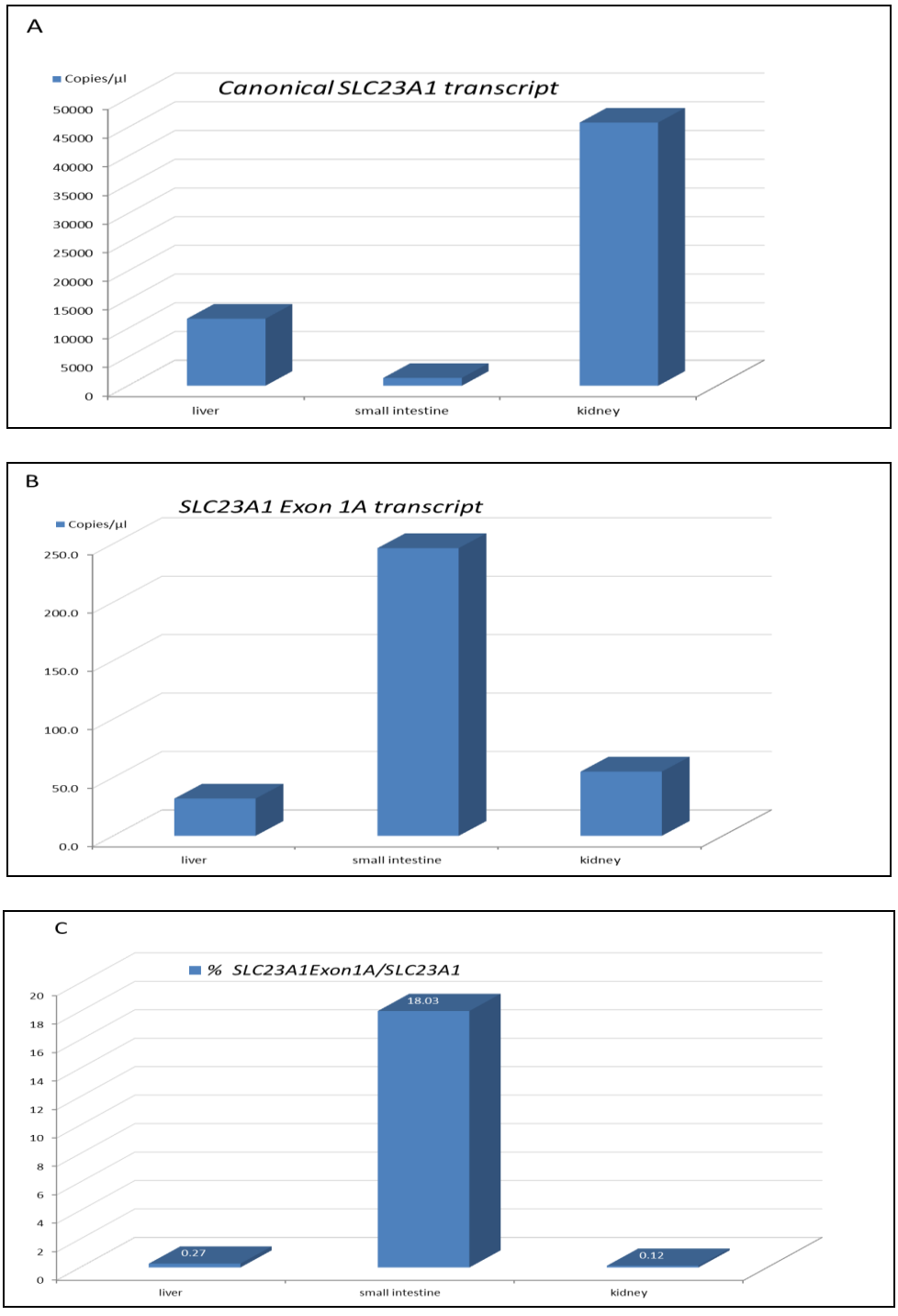


Figure 4.3: Expression of differentially spliced human SLC23A1 transcripts in liver, small intestine, and kidney. A) The consensus transcript is highly abundant in all tissues confirming prior observations (5, 11, 12, 16, 19). **B)** Exon 1A is only detectable in trace amounts in liver and kidney, but moderately expressed in the small intestine. **C)** 18.3% of transcripts in the small intestine contain exon 1A, while less than 0.3% of transcripts in liver and kidney contain exon 1A.

The expression pattern strongly indicates a tissue specific utilization of exon 1A, with significant abundance in the small intestine. The novel SLC23A1 exon 1A transcript has a total length of 2473 nucleotides (sequence in the appendix I, chapter IV).

4.4.2 THE NOVEL SLC23A1 PROTEIN ISOFORM ADDS 36 N-TERMINAL AMINO ACIDS BUT DOES NOT ALTER TRANSMEMBRANE TOPOLOGY OR INTRACELLULAR LOCATION

The novel *SLC23A1* Exon 1A contains a translation initiation codon (ATG) **(Figure 4.1A)**, defining the start of a 1905 nucleotide Open Reading Frame (ORF) (Sequence in the supplement). The resulting novel 634 amino acids containing protein, we name it SLC23A1 isoform A, is in frame with the previously described 598 amino acid containing SLC23A1 protein (NP689898 (5, 11)), adding 36 additional N-terminal amino acids (Supplement Figure 1, and protein sequences).

The novel SLC23A1 isoform 1A containing 634 amino acids has a predicted molecular weight of 68.9 kDa. The additional 36 N-terminal amino acid are predicted to change the ratio of negatively charged to positively charged amino acids from 41/37 to 41/44, respectively, changing the charge of the protein from -4 to +3. The theoretical pI is changed from 6.16 for the short isoform B (NP689898) to 8.09 for the novel longer isoform A. Both proteins are predicted to be stable and aliphatic index and grand average of hydropathicity are similar. Furthermore, no changes in membrane topology are predicted, with the N- and the C-termini predicted to be cytosolic **(Figure 4.4)**.

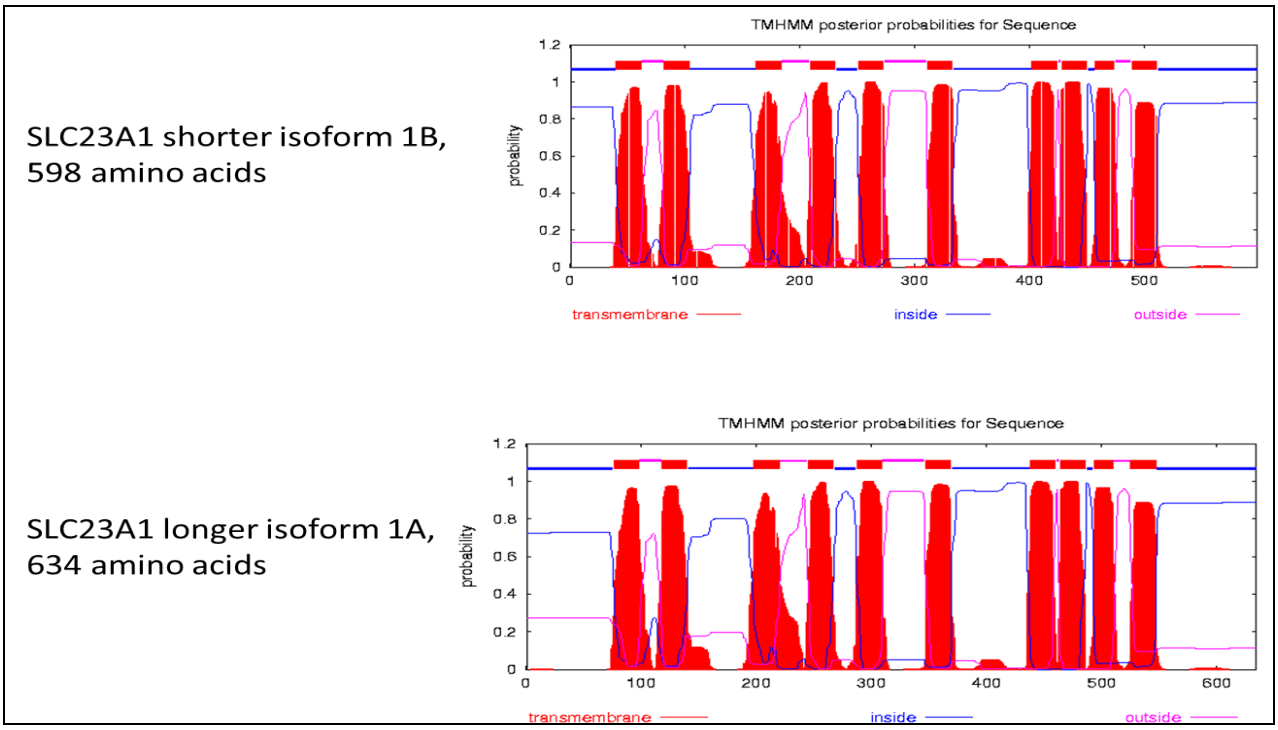


Figure 4.4: Predicted membrane topology for the SLC23A1 protein isoforms.
A) The reference protein NP689898 represents the shorter SLC23A1 isoform 1B, and 10 transmembrane domains are predicted. **B)** The additional 36 nucleotides in the novel SLC23A1 isoform 1A are not predicted to change the membrane topology, but add to the length of the N-terminal region.

Three novel protein-protein binding sites are predicted in the novel N-terminus of SLC23A1 protein isoform 1A (Appendix I, chapter IV Figure 2), and two additional protein kinase C phosphorylation sites are predicted, indicating an altered activation pattern for this isoform (Appendix I, chapter IV Figure 2). No signal peptide sequences are identified in both isoform, indicating similar sub-cellular location.

4.4.3 THE NOVEL SLC23A1 PROTEIN ISOFORM 1A LOCATES TO THE PLASMA MEMBRANE

When transiently expressed in human embryonic kidney cells (HEK 293), the shorter SLC23A1 isoform B locates on the outer plasma membrane **(Figure 4.5A)**, as has been described before (9, 20). The longer SLC23A1 isoform A shows the same membrane localization **(Figure 4.5B)**. Both isoforms exhibit strong expression when they are solely expressed in the HEK293 cells.

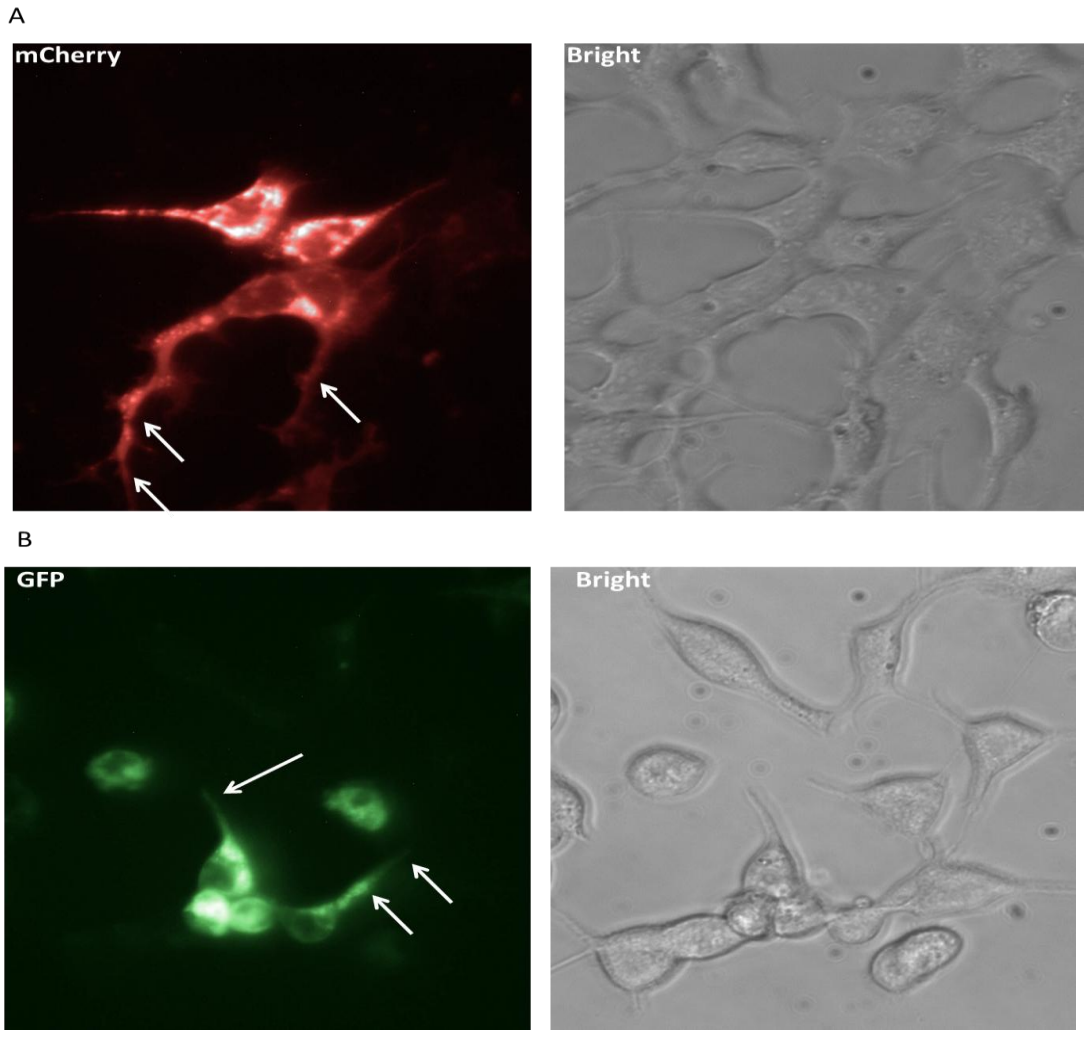


Figure 4.5: **Localization of the two isoforms. A)** mCherry (red) tagged SLC23A1 isoform 1B locates to the plasma membrane in HEK 293 cells. **B)** tGFP (green) tagged SLC23A1 isoform 1A also locates to the plasma membrane in HEK 293 cells.

4.4.4 THE NOVEL SLC23A1 PROTEIN ISOFORM 1A MEDIATES VERY LOW ASCORBIC ACID TRANSPORT IN *XENOPUS LAEVIS* OOCYTES

When expressed in *Xenopus laevis* oocytes the novel SLC23A1 isoform 1A mediated about 7% of the transport capacity of isoform B **(Figure 4.6)**.

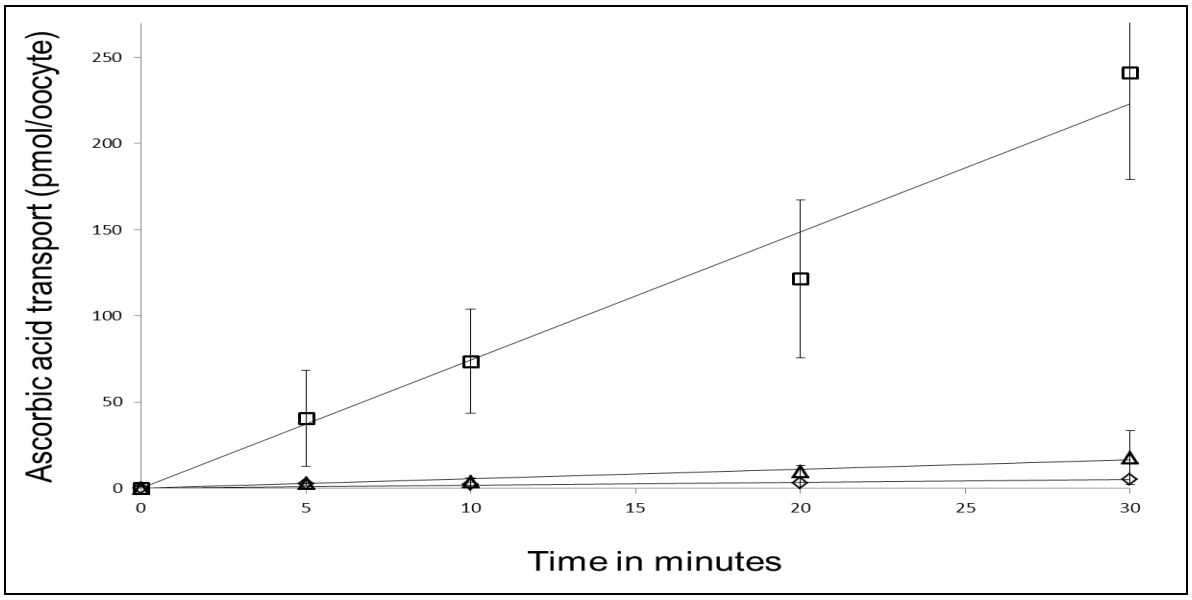


Figure 4.6: Transport activity of the two isoforms. The novel SLC23A1 isoform 1A mediates ascorbic acid uptake in Xenopus laevis oocytes. [^{14}C]ascorbic acid uptake into Xenopus laevis oocytes injected with cRNA encoding SLC23A1 isoform 1A (Δ) is elevated compared to sham injected oocytes (◊), however, it is only about 7% compared to SLC23A1 isoform 1B (□), when incubated with 300 μM [^{14}C] ascorbic acid.

4.4.5 DEFINITION OF THE TRANSCRIPTIONAL START POINT OF *SLC23A1* EXON 1B

EST alignments in the 5' region of *SLC23A1* exon 1B shows multiple transcripts extending beyond the previously described transcriptional start point defined by reference RNAs NM005847.4 and NM152685.3 **(Figure 4.1B)** (13, 14). PCR amplification of RNA from human

intestinal tissue confirm a 144 nucleotides spanning exon 1B, including a 108 nucleotides 5' untranslated region (5'UTR) **(Figure 4.1B)**. The total size of the transcript of the shorter *SLC23A1* splice variant 1B is therefore 2355 nucleotides from TSP to poly-A signal (sequence in the appendix I chapter IV).

4.5 DISCUSSION

This report describes a previously uncharacterized alternative first exon of the *SLC23A1* gene, called SLC23A1 exon 1A, which is located 1078 nucleotides 5' of the currently known *SLC23A1* exon 1B and is preferably expressed in the small intestine. It encodes a protein isoform that contains additional 36 N-terminal amino acids, locates to the outer cell membrane, but mediates only about 7% of the ascorbic acid transport of the shorter isoform. The reason for this lack of transport activity is currently not apparent, since both isoforms are predicted to have the same membrane architecture and the molecular imaging confirmed that both locate to the outer cell membrane in mammalian cells. However, the lack of transport activity might be explained by two mechanisms. First, the net charges of the two proteins are reversed, from -4 to +3 and the pI is significantly altered from 6.16 to 8.09 between isoform 1B and isoform 1A, respectively. Therefore simple structural alteration might cause the decreased function. Secondly, protein activation could be modified by two additional protein kinase C (PKC) phosphorylation sites and three novel protein-protein binding sites in the additional N-terminus. PKC phosphorylation had been shown to inhibit membrane proteins activity through decreased structural flexibility as well as modifying the effects of other protein kinases such as protein kinase G and protein kinase A

(21). In addition, PKC activity had been shown to downregulate SLC23A1 via derecruitment from the plasma membrane (22,23).

The existence of the less functional SLC23A1 isoform A might indicate redundancy, specifically since the locus is not evolutionary conserved and the transcript for *SLC23A1* variant A is only found in humans. The simple lack of strong evolutionary pressures to decrease the size of the human transcriptome might explain the existence of a less functional membrane transporter isoform (24). However, this theory might not apply to a human intestinal ascorbic acid transporter, since primates lost the ability to synthesize ascorbic acid and solely rely on intestinal absorption. Therefore the recently developed concept that transporter efficiency is fine-tuned to specific ranges of substrate concentration offers an explanation for the existence of SLC23A1 isoform 1A (24). Since ascorbic acid concentrations in the small intestine vary greatly, the amino acid composition of the different isoforms might be adapted to provide optimal substrate/transporter interactions. Substrate concentrations in the small intestine can exceed 300 µM, which was tested in the presented experiments, and isoform A might have better efficiency in higher substrate concentrations, where isoform B reaches maximal saturation (5, 11, 12, 25). This would also explain the lack of SLC23A1 isoform A in the kidney and liver, where maximal ascorbic acid concentrations do not exceed 100 µM, matching the affinity and capacity of SLC23A1 isoform 1B (11), rendering an additional isoform obsolete. This would be consistent with the general theory that alternative splicing multiplies the encoding volume of any given genome of eukaryotes and the differential regulation of alternative splice variants is considered to play a substantial role in defining the phenotype of a given cell type, or cell state (18).

To conclude, this is the first report of an alternative splice variant since the initial description of the *SLC23A1* transcript (11, 12, 26) or the characterization of the gene locus (13, 14). The novel alternative protein isoform A exhibits low ascorbic acid transport activity at a substrate concentration of 300 μM, however, its specific roles and possible synergism with the more functional SLC23A1 isoform 1B remains to be determined. Moreover, the human SLC23A1 locus extends about 1kb further 5' than previously described (13, 14).

4.6 REFERENCES

1. Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., et al. 2003. Vitamin C as an antioxidant: Evaluation of its role in disease prevention. *Journal of the American College of Nutrition* 22:18-35.

2. Mandl, J., Szarka, A., and Bánhegyi, G. 2009. Vitamin C: update on physiology and pharmacology. *British Journal of Pharmacology* 157:1097-1110.

3. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U.V., Chen, X.Z., Wang, Y., Brubaker, R.F., and Hediger, M.A. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 399:70-75.

4. Daruwala, R., Song, J., Koh, W.S., Rumsey, S.C., and Levine, M. 1999. Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. *FEBS Lett* 460:480-484.

5. Wang, H., Dutta, B., Huang, W., Devoe, L.D., Leibach, F.H., Ganapathy, V., and Prasad, P.D. 1999. Human Na(+)-dependent vitamin C transporter 1 (hSVCT1): primary structure, functional characteristics and evidence for a non-functional splice variant. *Biochim Biophys Acta* 1461:1-9.

6. Wang, Y., Mackenzie, B., Tsukaguchi, H., Weremowicz, S., Morton, C.C., and Hediger, M.A. 2000. Human vitamin C (L-ascorbic acid) transporter SVCT1. *Biochem Biophys Res Commun* 267:488-494.

7. Boyer, J.C., Campbell, C.E., Sigurdson, W.J., and Kuo, S.M. 2005. Polarized localization of vitamin C transporters, SVCT1 and SVCT2, in epithelial cells. *Biochemical and Biophysical Research Communications* 334:150-156.

8. Subramanian, V.S., Marchant, J.S., and Said, H.M. 2010. Molecular determinants dictating cell surface expression of the human sodium-dependent vitamin C transporter-2 in human liver cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 298:G267-G274.

9. Varma, S., and Kuo, S.M. 2007. Essential role of a conserved transmembrane histidine residue in the activity of two homologous sodium-dependent vitamin C transporters, SVCT1 and SVCT2. *FASEB Journal* 21:A244-A244.

10. Corpe, C.P., Lee, J.H., Kwon, O., Eck, P., Narayanan, J., Kirk, K.L., and Levine, M. 2005. 6-Bromo-6-deoxy-L-ascorbic acid: an ascorbate analog specific for Na+-dependent vitamin C transporter but not glucose transporter pathways. *J Biol Chem* 280:5211-5220.

11. Daruwala, R., Song, J., Koh, W.S., Rumsey, S.C., and Levine, M. 1999. Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. *FEBS Letters* 460:480-484.

12. Wang, Y.X., Mackenzie, B., Tsukaguchi, H., Weremowicz, S., Morton, C.C., and Hediger, M.A. 2000. Human vitamin C (L-ascorbic acid) transporter SVCT1. *Biochemical and Biophysical Research Communications* 267:488-494.

13. Erichsen, H.C., Eck, P., Levine, M., and Chanock, S. 2001. Characterization of the genomic structure of the human vitamin C transporter SVCT1 (SLC23A2). *Journal of Nutrition* 131:2623-2627.

14. Eck, P., Erichsen, H.C., Taylor, J.G., Yeager, M., Hughes, A.L., Levine, M., and Chanock, S.J. 2004. Comparison of the genomic structure and variation in the two human sodium-dependent vitamin C transporters, SLC23A1 and SLC23A2. *Human Genetics* 115:285-294.

15. Gispert, S., Dutra, A., Lieberman, A., Friedlich, D., and Nussbaum, R.L. 2000. Cloning and genomic organization of the mouse gene Slc23a1 encoding a vitamin C transporter. *DNA Research* 7:339-345.

16. Corpe, C.P., Tu, H., Eck, P., Wang, J., Faulhaber-Walter, R., Schnermann, J., Margolis, S., Padayatty, S., Sun, H., Wang, Y., et al. 2010. Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice. *Journal of Clinical Investigation* 120:1069-1083.

17. Sammeth, M., Foissac, S., and Guigo, R. 2008. A general definition and nomenclature for alternative splicing events. *PLoS Comput Biol* 4:e1000147.

18. Sammeth, M., Foissac, S., and Guigo, R. 2008. A General Definition and Nomenclature for Alternative Splicing Events. *Plos Computational Biology* 4.

19. Tsukaguchi, H., Tokui, T., Mackenzle, B., Berger, U.V., Chen, X.Z., Wang, Y., Brubaker, R.F., and Hediger, M.A. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 399:70-75.

20. Varma, S., Sobey, K., Campbell, C.E., and Kuo, S.M. 2009. Hierarchal contribution of N- and C-terminal sequences to the differential localization of homologous sodium-dependent vitamin C transporters, SVCT1 and SVCT2, in epithelial cells. *Biochemistry* 48:2969-2980.

21. Zhou, X.B., Wulfsen, I., Utku, E., Sausbier, U., Sausbier, M., Wieland, T., Ruth, P., and Korth, M. 2010. Dual role of protein kinase C on BK channel regulation. *Proceedings of the National Academy of Sciences of the United States of America* 107:8005-8010.

22. Shawki, A., Illing, A.C., Worrell, R.T., and Mackenzie, B. 2008. PKC activation downregulates the human Na+/L-ascorbic acid transporter SVCT1 via its derecruitment from the plasma membrane. *FASEB Journal* 22.

23. Samluk, L., Czeredys, M., Skowronek, K., and Nalecz, K.A. 2012. Protein kinase C regulates amino acid transporter ATB(0,+). *Biochemical and Biophysical Research Communications* 422:64-69.

24. Berezhkovskii, A.M., Lizunov, V.A., Zimmerberg, J., and Bezrukov, S.M. 2011. Functional Role for Transporter Isoforms in Optimizing Membrane Transport. *Biophysical Journal* 101:L14-L16.

25. Eck, P., Kwon, O., Wang, J., Chen, S., Schnermann, J., and Levine, M. 2013. The sodium dependent ascorbic acid transporters SLC23A1 mediates apical uptake in the renal proximal tubule. *submitted*

26. Wang, H.P., Dutta, B., Huang, W., Devoe, L.D., Leibach, F.H., Ganapathy, V., and Prasad, P.D. 1999. Human Na+-dependent vitamin C transporter 1 (hSVCT1): primary structure, functional characteristics and evidence for a non-functional splice variant. *Biochimica Et Biophysica Acta-Biomembranes* 1461:1-9.

TRANSITION STATEMENT 2

Localized vitamin C deficiency has generally been reported in the patients with IBD. Ascorbic acid disparity may play a major role in intestinal inflammation and tissue damage in IBD patients. Deregulation in vitamin C transporters may result in redox imbalance and thus could modulate the susceptibility to IBD and/or severity of its complications. As such, the association of vitamin C transporters with IBD needs to be determined.

In the following manuscript we investigated the association between variations in the *SLC23A1* locus (encoding the intestinal ascorbic acid transporter protein) with IBD risk. Through genotyping tagging SNPs, in our existing Manitoban IBD cohort, we observed convincing association between SNPs in *SLC23A1* gene and Crohn's disease.

This is the first study to investigate association between vitamin C transporters with IBD and the first report to show the contribution of variation in *SLC23A1*gene to risk of IBD.

CHAPTER 5

MANUSCRIPT 3

This manuscript was published in the American Journal of Clinical Nutrition

(Am J Clin Nutr 2014; 99:378–83. Copyright © 2014)

POLYMORPHISMS IN THE SODIUM-DEPENDENT ASCORBATE TRANSPORTER GENE *SLC23A1* ARE ASSOCIATED WITH SUSCEPTIBILITY TO CROHN'S DISEASE

Mandana Amir Shaghaghi [1, 2], Charles N. Bernstein [3, 4], Alejandra Serrano León[1, 2], Hani El-Gabalawy [4], Peter Eck [1, 2]

Afflications: [1]Human Nutritional Sciences, [2] The Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, Manitoba, R3T 2N2. [3] University of Manitoba IBD Clinical and Research Centre, and [4] Department of Internal Medicine, University of Manitoba, Winnipeg, Canada, R3E 3P4

5.1 ABSTRACT

Crohn's disease (CD) and ulcerative colitis (UC) are two common inflammatory bowel diseases (IBD) associated with intestinal inflammation and tissue damage. Oxidative stress is suggested to play a major role in the initiation and progression of IBD. Supplementation of Vitamin C (ascorbate, ascorbic acid) has reduced oxidative stress in persons with IBD. The role of ascorbate transporters in IBD remains to be determined. *SLC23A1* is a major ascorbate transporter in the intestinal tract and some of its genetic variants have been associated with severely decreased ascorbate transport and lowered systemic levels. This study aimed to determine if common genetic variants in vitamin C transporter *SLC23A1* are associated with risk of IBD. Genomic DNA samples from patients with CD (n=162) and UC (n=149) from the Manitoba IBD Cohort Study and ethnically matched controls (n=142) were genotyped for three *SLC23A1* polymorphisms (rs6596473, rs33972313, rs10063949) using TaqMan Assays. Variation at rs10063949 (G allele for heterozygote and homozygote) was associated with increased susceptibility to CD (OR=2.54, 95% CI 1.38, 4.66; OR=4.72, 95% CI 2.53, 8.81, $p<0.0001$; respectively). A strong linkage disequilibrium (LD) was observed across the *SLC23A1*region (variation rs6596473 with rs10063949) for CD and UC (D´=0.94, D´=0.96; respectively). The risk alleles confirmed a haplotype (CGG) which is carried more in CD patients (65.3%, $p<0.0001$) compared to controls (43.5%). A genetic variant (rs10063949-G) in the *SLC23A1* ascorbate transporter locus was identified which is associated with an increased risk of CD in a Caucasian Canadian IBD cohort. The presented evidence that *SLC23A1* variations can modulate the risk of CD has implications for understanding ascorbate transport in CD patients and provides a novel opportunity toward individualized nutritional therapy for the patients carrying the disease associated genotype.

5.2 INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC), and results from the interface of environmental factors with an aberrant immune response in genetically susceptible individuals. IBD is accompanied by excessive production of reactive oxygen species which plays an important role in the pathogenesis of the disease through oxidative tissue damage (1). The antioxidant defense system of the intestinal mucosa is impaired in IBD patients which results in increased oxidative injury and eventually delayed recovery of the inflamed mucosa (2, 3). Intestinal biopsies from IBD patients have shown deficiencies and imbalances in the levels of different antioxidants, including Vitamin C (ascorbate) in inflamed mucosa compared with normal mucosa (2, 4).

Ascorbate is the primary essential water-soluble antioxidant from the diet which acts as direct scavenger of reactive oxygen species and contributes to prevention of oxidative damage. Ascorbate is also a redox cofactor for enzymes required for the synthesis of collagen, carnitine, and neurotransmitters (5-7). The antioxidant property of ascorbate is thought to prevent chronic diseases involving inflammatory events, including atherosclerosis (8-10), cancer (11), and type 2 diabetes (12, 13). In IBD patients, a loss of 35-73% total and reduced ascorbate has been observed in inflamed mucosa which contributes to the overall loss of its antioxidant capacity (4). Plasma deficiencies of ascorbate have also been observed in IBD patients (14-16) and has been attributed to extensive depletion of ascorbate as an antioxidant or to inadequate dietary uptake (17). Whether reduction in ascorbate levels in inflammatory tissue in IBD is more cause or effect is unknown.

The sodium-dependent ascorbate transporter 1 (SLC23A1) is the major ascorbate transporter in the intestinal epithelium (18, 19) and is also found in cells involved in the immune defense (20, 21). Cellular ascorbic acid uptake is enhanced when SLC23A1 is present (19, 22, 23). Global elimination of *slc23a1* in the mouse results in a dramatic decrease of ascorbic acid levels in cells and organs expressing *slc23a1* in the wild type (24). A non-synonymous genetic variation has been shown to reduce the transporters capacity by approximately 90% *in vitro* (24). We hypothesized that variation in the human *SLC23A1* gene down regulates transporter activity and reduces intracellular antioxidant capacity resulting in impeding the enterocytes barrier function and/or modulating some intestinal immune cells capability to respond to oxidative stress. Therefore, the objective of this study was to examine if genetic variation in *SLC23A1* gene could modulate the susceptibility to IBD or severity of its complications.

5.3 SUBJECTS AND METHODS

5.3.1 STUDY DESIGN AND POPULATION

The study design has previously been described (25). Briefly, clinical data were collected from case records of participants in the Manitoba IBD Cohort Study, initiated in 2002. At enrollment in the Cohort Study, participants were at least 18 yr of age (18-80 yr) and diagnosed within the previous 7 yr (median 4.3 years). Controls included healthy individuals with no chronic immune diseases or first degree relatives with chronic immune diseases. A total of 311 persons with IBD (CD n=162, UC n=149) and 142 healthy controls were studied. All the study population (cases and controls) were Caucasian. The diagnosis and extent of IBD was determined based on surgical, endoscopic, radiologic, and histologic data. Phenotype was assigned according to the

Montreal Classification (26). Every subject signed an informed consent, and this study was approved by Biomedical Research Ethics Board at University of Manitoba.

5.3.2 SNP SELECTION AND GENOTYPING METHODS

Extensive sequencing analysis of the pattern of common genetic variation of *SLC23A1* gene has previously been performed defining haplotype tagging SNPs for the gene (27). Subsequently, a haplotype-based approach was implemented and three SNPs in the *SLC23A1* gene were identified (rs6596473C>G, rs33972313 A>G, and rs10063949 G>A). Variants were selected because they are located in the 5', middle and 3' region of *SLC23A1* and based on potential functional affect (e.g. rs33972313 is non-synonymous and had been shown to influence plasma ascorbic acid levels).

Genomic DNA was isolated from peripheral white blood cells as previously described (29). Genotyping was performed for all subjects for three SNPs in *SLC23A1* using TaqMan Real-Time PCR Assays (Applied Biosystems, Foster City, CA, USA), the assay condition for each optimized assay are shown in **Table 5.1**. Approximately 8% blinded quality control samples (36 individuals) were assayed 4 times, which showed 100% concordance.

5.3.3 STATISTICAL ANALYSIS

Statistical analyses were performed using Statistical Analysis Systems software (SAS), version 9.3 (SAS Inc., Cary, NC, USA). All tests were two-sided and significant *p* values was set at

P<0.05. Each of the three SNPs was analyzed individually using allele frequencies and carriage rates for association with CD and UC. Binary logistic regression was used to estimate odds ratios (ORs) and 95% confidence interval (CIs) for association between genotypes and CD and UC risk, adjusted for age and gender. Allelic frequencies and genotype frequencies were determined in UC, CD and controls. The homozygous common genotype was considered as reference group. In alternative analyses, a dominant model of inheritance was used to compare risk in the combined group of homozygous rare and heterozygotes genotypes to risk among the common homozygous genotype. Based on phenotype, comparisons of genetic frequencies and allelic frequencies were determined between UC, CD and controls by means of a 2x2 contingency table and chi-squared test. Haplotypes were calculated using Haploview 4.2 (Broad Institute, Cambridge, MA, USA). Multinomial logistic regression was used for association between genotypes and phenotype for CD and UC patients.

Table 5.1: Primers used to examine the three single nucleotide polymorphism in SLC23A1 gene involved in this study[1]

dbSNP	CGF assay ID	Position	Alleles	TaqMan primers	TaqMan probes
rs6596473	A-007155	138738475bp	C/G	F: CATTGAGGCTGCCACTTGAC R: TGCCCATTTAGAGGATGCTAGACT	FAM: CCTATGGGCCTGAGACA VIC: CCTATGGGCGTGAGACA
rs33972313	001-1224	138743401bp	A/G	F: AGACCTCCAGTGCCTTCAGT R: GCAGCACGTCTGTCAAGGT	FAM: TCATGACCGTGTGGCT VIC:CATCATGACCATGTGGCT
rs10063949	A-006670	138747425bp	G/A	F: TTTGACCCAAGCCATGCAGATA R: GGCAGCTCAGACCAACCT	FAM: TTCTGCAAACTTGC VIC: TCTGCGAACTTGC

[1]Adopted from reference (28); CGF, Core genotyping facility; F, forward primer; R, reverse primer

5.4 RESULTS

One hundred sixty two patients with CD, 149 patients with UC, and 142 ethnically matched healthy controls were genotyped for three *SLC23A1* polymorphisms. Baseline characteristics of the study population are shown in **Table 5.2**.

Table 5.2: General characteristics of the study subjects

Parameters	**Crohn´s Disease (n=162)**	**Ulcerative Colitis (n= 149)**	**Controls (n=142)**
Gender			
Female	97 (59.9%)	87 (58.4%)	80 (56.3%)
Male	65 (40.1%)	62 (41.6%)	62 (43.7%)
Age at diagnosis			
A1(<17 yr)	17 (10.5%)	12 (8.1%)	-
A2 (17-40 yr)	101 (62.3%)	78 (52.3%)	-
A3 (>40 yr)	44 (27.2%)	59 (39.6%)	-
Location			
L1 (Ileal)	69 (42.6%)	-	-
L2 (Colonic)	37 (22.8%)	-	-
L3 (Ileocolonic)	51 (31.5%)	-	-
L4 (isolated upper disease)	5 (3.1%)	-	-
E1(Denotes proctitis)	-	11 (7.4%)	-
E2 (Left-sided)	-	68 (45.6%)	-
E3 (Extensive colitis)	-	70 (47.0%)	-
Behaviour			
B1(Inflammatory)	69 (42.6%)	-	-
B2 (Stricturing)	54 (33.3%)	-	-
B3 (Penetrating/fistulizing)	39 (24.1%)	-	-

Note: No significance difference was found between the base line characteristic for study populations.

Genotype frequencies are summarized in **Table 5.3** (CD) and **Table 5.4** (UC). Genotype frequencies for all of the polymorphisms were in Hardy-Weinberg equilibrium within CD and UC groups.

Among the 3 variants in *SLC23A1* examined, the rs10063949-G allele is associated with increased CD risk (**Table 5.3**). Carriage of one (heterozygote) or two (homozygote) copies of the minor rs10063949 allele (G) was associated with increased risk of CD [OR=2.54, 95% CI 1.38, 4.66; OR=4.72, 95% CI 2.53, 8.81, $p<0.001$; respectively). None of the SNPs were associated with UC risk.

Table 5.3: Genotype, allele, and haplotype frequencies of *SLC23A1* gene variants in Crohn's disease patients and control subjects

	Crohn´s disease n=162	**Controls n=142**	**OR (95% CI)**	***P***
rs6596473				
GG	77 (47.5%)	67 (47.2%)	REF	
CG	65 (40.1%)	64 (45.1%)	0.88 (0.55, 1.42)	0.61
CC	20 (12.3%)	11 (7.7%)	1.58 (0.71, 3.54)	0.26
C-carrier	105 (32.4%)	87 (30.6%)	1.09 (0.77, 1.53)	0.64
rs33972313				
GG	156 (96.3%)	138 (97.2%)	REF	
GA	6 (3.7%)	4 (2.8%)	1.33 (0.37, 4.08)	0.67
AA	0	0	ND	ND
A-carrier	6 (1.9%)	4 (1.4%)	1.32 (0.37, 4.73)	0.66
rs10063949				
AA	24 (14.8%)	53 (37.3%)	REF	
GA	61 (37.7%)	53 (37.3%)	2.54 (1.38, 4.66)	0.001
GG	77 (47.5%)	36 (25.4%)	4.72 (2.53, 8.81)	0.001
G-carrier	216 (66.7%)	125 (44%)	2.54 (1.83, 3.53)	0.001
Haplotype				
CGG	106 (65.3%)	62 (43.5%)	2.44 (1.54, 3.88)	0.0001

Odds ratios are adjusted for age and gender
Per allele effects are derived from binary logistic regression
ND = not determined
The haplotypes were formed by the SNPs rs6596473, rs33972313, and rs10063949

A strong linkage was observed across the *SLC23A1* gene (D' = 0.94 between rs6596473 and rs10063949) in persons with CD **(Figure 5.1A)** and the haplotype CGG which is carried in persons with CD (OR=2.40, 95% CI: 1.54, 3.88, *P*<0.0001) (**Table 5.3**). Strong linkage (D'= 0.96) in the SLC23A1 locus was also observed in UC patients for the two SNPs (rs6596473 and rs10063949) (**Figure 5.1B**) and a major haplotype (frequency ≥5%) was inferred for UC patients (CAG: 41%).

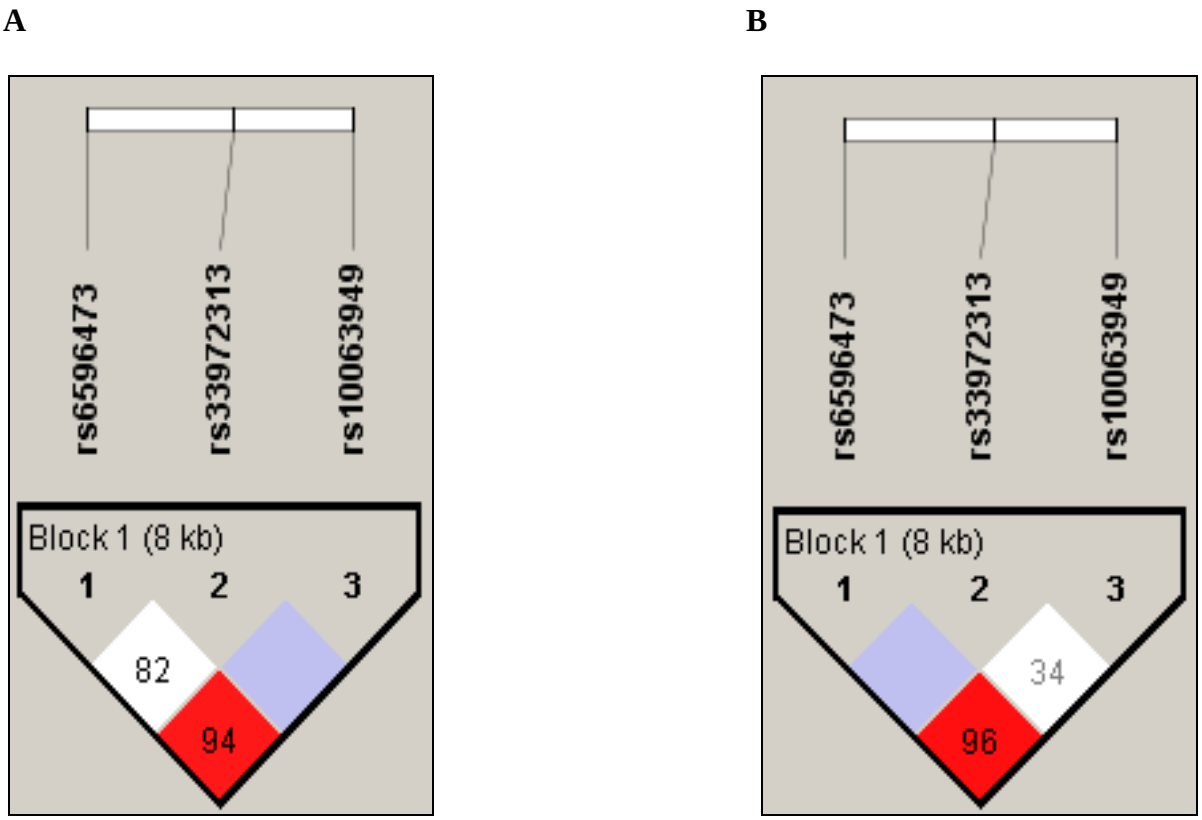


Figure 5.1: **Linkage observed across the SLC23A1 gene locus in individuals with Crohn's disease (A) and Ulcerative colitis (B).** The degree of linkage disequilibrium (LD) is given in percentage, represented in the triangles. Red indicates a high degree of LD, while blue indicates uncertain results.

No association was found between *SLC23A1* genotype and haplotype with UC risk (**Table 5.4**). No correlation was found between the presence of any genotype and specific phenotypes for CD and UC (data not shown).

Table 5.4: Genotype, allele, and haplotype frequencies of SLC23A1 gene variants in ulcerative colitis patients and control subjects

	Ulcerative Colitis n=149	**Controls n=142**	**OR (95% CI)**	***P***
rs6596473				
GG	66 (44.3%)	67 (47.2%)	REF	
CG	68 (45.6%)	65 (45.8%)	1.05 (0.65, 1.69)	0.85
CC	15 (10.1%)	11 (7.7%)	1.36 (0.58, 3.19)	0.47
C-carrier	98 (32.9%)	87 (30.6%)	1.11 (0.78, 1.57)	0.56
rs33972313				
GG	142 (95.3%)	138 (97.2%)	REF	
GA	7 (4.7%)	4 (2.8%)	1.70 (0.49, 5.94)	0.40
AA	0	0	ND	ND
A-carrier	7 (2.3%)	5 (1.8%)	1.34 (0.42, 4.27)	0.62
rs10063949				
AA	58 (38.9%)	53 (37.3%)	REF	
GA	52 (34.9%)	53 (37.3%)	0.90 (0.53, 1.53)	0.69
GG	39 (26.2%)	36 (25.4%)	0.99 (0.55, 1.78)	0.97
G-carrier	131 (43.9%)	125 (44%)	1.01 (0.72, 1.38)	0.98
Haplotype				
CAG	63 (42.6%)	60 (42.5%)	1.00 (0.63, 1.59)	0.93

Odds ratios are adjusted for age and gender
Per allele effects are derived from Binary logistic regression
ND = not determined
The haplotypes were formed by the SNPs rs6596473, rs33972313, and rs10063949

5.5 DISCUSSION

Here, for the first time, we identified a variation in the sodium-dependent ascorbic acid transporter gene *SLC23A1* that is associated with CD, but not with UC. This finding confirms our hypothesis that a disturbance in the antioxidant balance could contribute to the development and severity of IBD. The rs10063949-G allele in *SLC23A1* gene is associated with an increased CD risk. Of persons with CD, two thirds carried the G allele, corresponding to a CD risk which is elevated by 2.5 times compared to the rs10063949-A carriers. The rs10063949-GG genotype was significantly over transmitted in the CD patients (47.5%) compared to controls (25.4%). An allele dosage effect resembling haploinsufficiency is evident. Compared to rs10063949-AA homozygotes the 10063949-AG heterozygotes have a 2.5 fold elevated risk for CD and the 10063949-GG homozygotes have a 4.7 fold elevated CD risk. No relation was observed between genetic variants in *SLC23A1* and UC.

The SNP rs10063949 is located within the promoter region of the *SLC23A1* gene, which contains a variety of regulatory elements such as the hepatocyte nuclear factor 1 (HNF1), required for tissue specific transcription (30). Assuming that SNP rs10063949 contributes to intestinal inflammation, it would most likely act through differential regulation of *SLC23A1* expression, which consequently would result in reduced cellular accumulation of ascorbate. In the current study, finding a genetic alteration in *SLC23A1* transporter gene in CD suggests that the observation of low ascorbate levels in inflamed mucosa in the study by Head and colleagues (2) may be as much cause of inflammation as effect.

The implication of the association between SNP rs10063949 and CD but not UC is undetermined. We here suggest three possible cell-type specific mechanisms which could by themselves or in combination with each other cause the differential association. If one or all of them gets validated in future biological and clinical studies, SNP rs10063949 could be utilized as predictive biomarker for CD. Further it should be determined if it could also serve as a diagnostic biomarker for dietary intervention with ascorbate. Firstly, cell type specific transcription factors regulating *SLC23A1* expression in immune regulatory cells involved in the development of CD but not UC might be affected by SNP rs10063949. *SLC23A1* is expressed in lymphocytes, however, the specific expression during hematopoiesis and therefore in the different types of lymphocytes, and leukocytes in general, is not defined. Future genomic and functional studies should determine *SLC23A1* expression patterns in all lymphocyte types (including natural killer cells), to define if indeed ascorbate accumulates differently in carriers of the SNP 10063949 genotypes. The levels of ascorbate will determine the antioxidative capacity (11) and as a consequence would contribute to the differential regulation of gene expression influencing CD but not UC risk.

A second possible mechanism addresses a potential dysregulation of macrophage function through reduction in ascorbate uptake of macrophages in epithelioid granulomas. A granuloma is a collection of macrophages and other inflammatory cells. Epithelioid granulomas are among the most specific microscopic features of CD, distinguishing it from UC (31). Intracellular ascorbic acid has a regulatory role in the granulocyte macrophage-colony-stimulating (GM-CSF) signaling response (32), which is involved in proinflammatory processes, and therefore could determine the severity of CD.

A third possible explanation is related to the fact that the intestinal epithelium constitutes a physical barrier which contains immunogenic bacteria within the intestinal lumen and elevated oxidative stress can impede this barrier function (33, 34). *SLC23A1* is highly expressed in intestinal epithelial enterocytes, where its down-regulation may lead to decreased intracellular ascorbic acid levels to compromise the physical barrier function against immunogenic bacteria and/or the ability to withstand prolonged intrinsic macrophage challenges. This by itself may not explain the causality of a genetic variation associated to CD but not UC, however, it could enhance the severity of any immune dysregulations specific for CD. This might be even more potentiated by the fact that oxidative stress in epithelial cells and macrophages leads to the activation of the nuclear factor kappa B, an activator of pro-oxidative genes such as lipoxygenase, cycloxygenase-2, and inducible-nitric oxide synthase which leads to further elevated oxidative stress and reduced function of the intestinal barrier (35, 36).

Previously, the three SNPs examined in this study were shown to be associated with circulating plasma ascorbic acid, however the results are inconsistent (5, 37, 38). The inconsistency and/or heterogeneity among the results from different studies may be largely accounted for sensitivity of analyses in measuring plasma ascorbate, different assay protocols, and many confounding factors that often make the interpretation of observational data difficult (37). Therefore, there is a need for additional studies to confirm a possible relationship between *SLC23A1* genetic variations and circulating plasma ascorbic acid. In the current study, due to sampling collection methods, we were not able to include measurements of plasma ascorbic acid as a biomarker for ascorbate status. While this is a limitation of this study, we speculate that the association of the

genetic variation to CD may not influence plasma ascorbic acid levels, which are determined through the renal re-absorption rather than intestinal absorption (19).

Compared to current genome wide association studies (GWAS) our Manitoban population-based case-control cohort could be considered suboptimal in terms of sample size (39). However, a previous study of the Manitoba IBD Cohort (40) found significant associations for some of the previously described IBD-associated SNPs identified by GWAS in other large population based cohorts . Therefore, these results from a well phenotyped cohort of moderate size are noteworthy, but nonetheless should be reproduced in other cohorts.

If, as we hypothesize, intracellular ascorbate levels of specific intestinal cell types will be decrease by the action of SNPs in *SLC23A1*, a supplementation with dehydroascorbate would be worthy of study as the therapy of choice to compensate for this shortfall. Dehydroascorbate, the oxidized form of Vitamin C, does not exist under physiologic conditions (41, 42). However, if supplemented externally, dehydroascorbate enters the cell through facilitated glucose transporter of the GLUT family, not SLC23A1 (23), and intracellular dehydroascorbate is immediately reduced to ascorbate, the active form of vitamin C (41-43). As currently dehydroascorbate is a minor component of some dietary supplements, the proposed gene specific personalized nutritional therapy would boost intracellular vitamin C levels and be considered safe. Confirmation of our findings in independent association studies is warranted before implementing any nutritional intervention.

5.6 ACKNOWLEDGMENTS

The authors gratefully acknowledge the participants of the Manitoba IBD Cohort Study. The authors' responsibilities were as follows—MAS: was the principal manuscript author and was responsible for laboratory analysis, data collection, and statistical analysis; CNB: was responsible for conducting the Manitoba IBD Cohort Study (funded by the Canadian Institutes of Health Research), sample collection, and contributed to revisions of the final manuscript; ASL: was contributed to interpreting data and to revise the final manuscript; HEC: was responsible for sample collection for healthy controls and contributed to revisions of the final manuscript; PE: was the principal investigator and responsible for the conception and design of the project, seeking financial support and contribution to the preparation of the manuscript. All the authors reviewed the final version of the manuscript and MAS, ASL, HEC, and PE had no conflicts of interest to declare. Dr. Bernstein has served on the advisory boards or has consulted to Abbott Canada, Abbvie Canada, Bristol Myers Squibb, Forest Canada, Takeda Canada, Janssen Canada, Hospira, and Vertex Pharmaceuticals and has received research grants from Abbott Canada and Abbvie Canada and an education grant from Aptalis. He is supported in part by the Bingham Chair in Gastroenterology.

5.7 REFERENCES

1. Kruidenier, L., Kuiper, I., Lamers, C.B., and Verspaget, H.W. 2003. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 201:28-36.

2. Head, K.A., and Jurenka, J.S. 2003. Inflammatory bowel disease Part 1: ulcerative colitis--pathophysiology and conventional and alternative treatment options. *Altern Med Rev* 8:247-283.

3. Kruidenier, L., and Verspaget, H.W. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 16:1997-2015.

4. Buffinton, G.D., and Doe, W.F. 1995. Altered ascorbic acid status in the mucosa from inflammatory bowel disease patients. *Free Radic Res* 22:131-143.

5. Cahill, L.E., and El-Sohemy, A. 2009. Vitamin C transporter gene polymorphisms, dietary vitamin C and serum ascorbic acid. *J Nutrigenet Nutrigenomics* 2:292-301.

6. Carr, A.C., and Frei, B. 1999. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. *Am J Clin Nutr* 69:1086-1107.

7. Garg, L.M., Wood, L. G. 2013. *Nutrition & Physical Activity in Inflammatory Diseases*. Boston, USA: CABI.

8. Polidori, M.C., Mecocci, P., Levine, M., and Frei, B. 2004. Short-term and long-term vitamin C supplementation in humans dose-dependently increases the resistance of plasma to ex vivo lipid peroxidation. *Arch Biochem Biophys* 423:109-115.

9. Jialal, I., Vega, G.L., and Grundy, S.M. 1990. Physiologic levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis* 82:185-191.

10. Balkan, J., Dogru-Abbasoglu, S., Aykac-Toker, G., and Uysal, M. 2004. Serum pro-oxidant-antioxidant balance and low-density lipoprotein oxidation in healthy subjects with different cholesterol levels. *Clin Exp Med* 3:237-242.

11. Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., et al. 2003. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J Am Coll Nutr* 22:18-35.

12. Salmeron, J., Manson, J.E., Stampfer, M.J., Colditz, G.A., Wing, A.L., and Willett, W.C. 1997. Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. *JAMA* 277:472-477.

13. Harding, A.H., Wareham, N.J., Bingham, S.A., Khaw, K., Luben, R., Welch, A., and Forouhi, N.G. 2008. Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: the European prospective investigation of cancer--Norfolk prospective study. *Arch Intern Med* 168:1493-1499.

14. Hengstermann, S., Valentini, L., Schaper, L., Buning, C., Koernicke, T., Maritschnegg, M., Buhner, S., Tillinger, W., Regano, N., Guglielmi, F., et al. 2008. Altered status of antioxidant vitamins and fatty acids in patients with inactive inflammatory bowel disease. *Clin Nutr* 27:571-578.

15. Sakamoto, N., Kono, S., Wakai, K., Fukuda, Y., Satomi, M., Shimoyama, T., Inaba, Y., Miyake, Y., Sasaki, S., Okamoto, K., et al. 2005. Dietary risk factors for inflammatory bowel disease: a multicenter case-control study in Japan. *Inflamm Bowel Dis* 11:154-163.

16. Hoffenberg, E.J., Deutsch, J., Smith, S., and Sokol, R.J. 1997. Circulating antioxidant concentrations in children with inflammatory bowel disease. *Am J Clin Nutr* 65:1482-1488.

17. Ioannidis, O., Varnalidis, I., Paraskevas, G., and Botsios, D. 2011. Nutritional modulation of the inflammatory bowel response. *Digestion* 84:89-101.

18. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U.V., Chen, X.Z., Wang, Y., Brubaker, R.F., and Hediger, M.A. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 399:70-75.

19. Corpe, C.P., Tu, H., Eck, P., Wang, J., Faulhaber-Walter, R., Schnermann, J., Margolis, S., Padayatty, S., Sun, H., Wang, Y., et al. 2010. Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice. *J Clin Invest* 120:1069-1083.

20. Unigene. 2013. EST Profile: Hs.643467 - SLC23A1: Solute carrier family 23 (nucleobase transporters), member 1. NCBI.

21. NCBI. 2013. Metastatic melanoma: peripheral blood lymphocytes; Organism, Homo sapiens; Profile GDS2735 / 14605 / SLC23A1.

22. Song, J., Kwon, O., Chen, S., Daruwala, R., Eck, P., Park, J.B., and Levine, M. 2002. Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and glucose. *Journal of Biological Chemistry* 277:15252-15260.

23. Corpe, C.P., Lee, J.H., Kwon, O., Eck, P., Narayanan, J., Kirk, K.L., and Levine, M. 2005. 6-Bromo-6-deoxy-L-ascorbic acid: an ascorbate analog specific for Na+-dependent vitamin C transporter but not glucose transporter pathways. *J Biol Chem* 280:5211-5220.

24. Corpe, C.P., Tu, H., Eck, P., Wang, J., Faulhaber-Walter, R., Schnermann, J., Margolis, S., Padayatty, S., Sun, H., Wang, Y., et al. 2010. Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice. *Journal of Clinical Investigation* 120:1069-1083.

25. Ediger, J.P., Walker, J.R., Graff, L., Lix, L., Clara, I., Rawsthorne, P., Rogala, L., Miller, N., McPhail, C., Deering, K., et al. 2007. Predictors of medication adherence in inflammatory bowel disease. *Am J Gastroenterol* 102:1417-1426.

26. Silverberg, M.S., Satsangi, J., Ahmad, T., Arnott, I.D., Bernstein, C.N., Brant, S.R., Caprilli, R., Colombel, J.F., Gasche, C., Geboes, K., et al. 2005. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 19 Suppl A:5-36.

27. Eck, P., Erichsen, H.C., Taylor, J.G., Yeager, M., Hughes, A.L., Levine, M., and Chanock, S.J. 2004. Comparison of the genomic structure and variation in the two human sodium-dependent vitamin C transporters, SLC23A1 and SLC23A2. *Human Genetics* 115:285-294.

28. Packer, B.R., Yeager, M., Staats, B., Welch, R., Crenshaw, A., Kiley, M., Eckert, A., Beerman, M., Miller, E., Bergen, A., et al. 2004. SNP500Cancer: a public resource for sequence validation and assay development for genetic variation in candidate genes. *Nucleic Acids Res* 32:D528-532.

29. Cantor, M.J., Nickerson, P., and Bernstein, C.N. 2005. The role of cytokine gene polymorphisms in determining disease susceptibility and phenotype in inflammatory bowel disease. *Am J Gastroenterol* 100:1134-1142.

30. Michels, A.J., and Hagen, T.M. 2009. Hepatocyte nuclear factor 1 is essential for transcription of sodium-dependent vitamin C transporter protein 1. *Am J Physiol Cell Physiol* 297:C1220-1227.

31. Pierik, M., De Hertogh, G., Vermeire, S., Van Assche, G., Van Eyken, P., Joossens, S., Claessens, G., Vlietinck, R., Rutgeerts, P., and Geboes, K. 2005. Epithelioid granulomas, pattern recognition receptors, and phenotypes of Crohn's disease. *Gut* 54:223-227.

32. Carcamo, J.M., Borquez-Ojeda, O., and Golde, D.W. 2002. Vitamin C inhibits granulocyte macrophage-colony-stimulating factor-induced signaling pathways. *Blood* 99:3205-3212.

33. John, L.J., Fromm, M., and Schulzke, J.D. 2011. Epithelial Barriers in Intestinal Inflammation. *Antioxidants & Redox Signaling* 15:1255-1270.

34. Christophi, G.P., Rong, R., Holtzapple, P.G., Massa, P.T., and Landas, S.K. 2012. Immune markers and differential signaling networks in ulcerative colitis and Crohn's disease. *Inflammatory Bowel Diseases* 18:2342-2356.

35. Maccarrone, M., Meloni, C., Manca-di-Villahermosa, S., Cococcetta, N., Casciani, C.U., Finazzi-Agrò, A., and Taccone-Gallucci, M. 2001. Vitamin E suppresses 5-lipoxygenase-mediated oxidative stress in peripheral blood mononuclear cells of hemodialysis patients regardless of administration route. *American Journal of Kidney Diseases* 37:964-969.

36. Pathak, S.K., Sharma, R.A., Steward, W.P., Mellon, J.K., Griffiths, T.R.L., and Gescher, A.J. 2005. Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies. *European Journal of Cancer* 41:61-70.

37. Timpson, N.J., Forouhi, N.G., Brion, M.J., Harbord, R.M., Cook, D.G., Johnson, P., McConnachie, A., Morris, R.W., Rodriguez, S., Luan, J., et al. 2010. Genetic variation at the SLC23A1 locus is associated with circulating concentrations of L-ascorbic acid (vitamin C): evidence from 5 independent studies with >15,000 participants. *Am J Clin Nutr* 92:375-382.

38. Zanon-Moreno, V., Ciancotti-Olivares, L., Asencio, J., Sanz, P., Ortega-Azorin, C., Pinazo-Duran, M.D., and Corella, D. 2011. Association between a SLC23A2 gene variation, plasma vitamin C levels, and risk of glaucoma in a Mediterranean population. *Mol Vis* 17:2997-3004.

39. Bush, W.S., and Moore, J.H. 2012. Chapter 11: Genome-wide association studies. *PLoS Comput Biol* 8:e1002822.

40. Ryan, J.D., Silverberg, M.S., Xu, W., Graff, L.A., Targownik, L.E., Walker, J.R., Carr, R., Clara, I., Miller, N., Rogala, L., et al. 2013. Predicting complicated Crohn's disease and surgery: phenotypes, genetics, serology and psychological characteristics of a population-based cohort. *Aliment Pharmacol Ther* 38:274-283.

41. Washko, P.W., Wang, Y., and Levine, M. 1993. Ascorbic acid recycling in human neutrophils. *Journal of Biological Chemistry* 268:15531-15535.

42. Wang, Y., Russo, T.A., Kwon, O., Chanock, S., Rumsey, S.C., and Levine, M. 1997. Ascorbate recycling in human neutrophils: Induction by bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 94:13816-13819.

43. Corpe, C.P., Eck, P., Wang, J., Al-Hasani, H., and Levine, M. 2013. Intestinal Dehydroascorbic acid (DHA) transport mediated by the facilitative sugar transporters, GLUT2 and GLUT8. *Journal of Biological Chemistry*.

TRANSITION STATEMENT 3

As one of the focal points of this research was to recognize new candidate transporter for vitamin C and further investigate on their contribution to IBD risk, we got interested to study GLUT 14, an orphan transporter.

SLC2A14 gene is located on human chromosome 12p13.31 and encodes GLUT 14, the recent identified member of the protein family of facilitative hexose transporters (GLUTs). GLUT 14 is specified only in human and described as a duplication of GLUT 3, however, the information about the gene structure is very limited. Based on the homology of GLUT 14 to GLUT 3, which is an active transporter for DHA (oxidized form of vitamin C); we speculate that GLUT 14 is also an enterocyte bound DHA transporter. We have, therefore, investigated the organization of the *SLC2A14* gene *in silico,* delineating its promoter region and splice variants.

Mapping the transcripts to the genome sequence, a significant expansion of the gene locus as well as tissue specific exon utilization was revealed. It further examined the sub-localization of the main protein isoforms and identified early evidence that GLUT 14 is a membrane transporter.

The results of this report facilitate further studies to clarify the regulation of *SLC2A14* expression and to assess whether mutations involving *SLC2A14* contribute to IBD.

CHAPTER 6

MANUSCRIPT 4

This manuscript is under revision by the Biochemistry and Cell Biology

SLC2A14 GENE: LOCUS, TISSUE EXPRESSION, SPLICE VARIANTS, AND SUBCELLULAR LOCALIZATION OF THE PROTEIN

Mandana Amir Shaghaghi [1], Brent Murphy [2], Peter Eck [1]

Afflications: [1]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada, R3T 2N2, [2] Department of Plant Science, University of Manitoba, Winnipeg, Canada.

6.1 ABSTRACT

The *SLC2A14* gene encodes for GLUT14, an orphan member of the facilitated membrane glucose transporter family, which was originally described to be exclusively expressed in human testis. However, genetic variations in *SLC2A14* are associated to chronic diseases such as Alzheimer's disease and inflammatory bowel disease, which cannot be explained by a strictly testicular expression. Therefore, we analyzed available information on the *SLC2A14* gene to update the knowledge on the locus and its encoded products. This report represents an expanded *SLC2A14* gene locus and a more diverse tissue expression, concurring with existing evidence of disease associations. The exon utilization is tissue specific, with major expression in testis. When the two major testicular protein isoforms were expressed in mammalian cells, they located to the plasmalemma membrane, providing early evidence that GLUT14 could have a function as a membrane transporter.

6.2 INTRODUCTION

The *SLC2A14* gene encodes for GLUT14, which was described as a specific to human and an orphan member of the SLC2/GLUT family of facilitated membrane hexose transporter (1). In 2002, Wu and Freeze described the *SLC2A14* gene structure, showing high homology on the transcript level to *SLC2A3* (GLUT3) (1). GLUT 3 has high affinity and high transport capacity for glucose and it also mediates cellular uptake of dehydroascorbic acid, the oxidized form of Vitamin C. Based on the high homology between GLUT14 with GLUT3, a function as facilitate glucose transporter is deduced for GLUT14 (2), however, this remains to be experimentally proven.

GLUT 14 was first described to be exclusively expressed in testis (1), however, further evidence indicates its expression in other tissues and cell lines, such as the ovarian cancer cell line A2780, where expression is downregulated in clonal cell lines resistant to the anticancer agents paclitaxel and cisplatin (3, 4). Abundance of the GLUT 14 transcript is also reported in the acute lymphoblastic leukemia cell lines NALM-6 and Jurkat (5), as well as in peripheral blood of Parkinson's disease patients, where it was upregulated compared to healthy controls (6). Taken together, this indicates that GLUT14 is expressed in tissues other than testis and could play a role in the development of common disease such as leukemia and Parkinson's disease.

Variations in the *SLC2A14* locus are implicated in common diseases of the central nervous system (7, 8), lymphatic cancer (5), rheumatoid arthritis (9), and intraocular pressure in primary open-angle glaucoma (10). Specifically, the single nucleotide polymorphism (SNP) rs10845990 is found to be associated with Alzheimer's disease in a Caucasian cohort (7), and carriers of the rs10845990-G allele showed a 41% higher risk for late-onset Alzheimer's disease in a Chinese cohort (8). Moreover, a deletion of 129 kilobytes in the chromosome 12p13.31 region, which includes the *SLC2A14* gene, confers a protective affect against rheumatoid arthritis across two Caucasian cohorts (9). A copy number variation in the 12p13.3 locus, overlapping the genes *SLC2A14* and *SLC2A3*, associated with intraocular pressure in glaucoma (10).

Despite emerging evidence that genetic variations in *SLC2A14* are associated with common and complex diseases, major gaps in our knowledge of the genomic locus, its transcripts, and encoded protein isoforms exist. In this paper we revisit the *SLC2A14* gene to present novel

information on the exon structure and utilization, alternative splicing, tissue expression, and intracellular localization of the two canonical protein isoforms.

6.3 MATERIAL AND METHODS

6.3.1 BIOINFORMATICS

Sequences. The National Center for Biotechnology Information (NCBI) database was used to obtain the genomic sequence (GRCh37.p13 (GCF_000001405.25); NC_000012.11 (7965110.8043792, complement) and the reference and non-reference RNAs for GLUT14. In addition, the Basic Local Alignment Search Tool (BLAST) was used to screen for additional human non-reference RNAs as well as expressed sequence tags (ESTs). Based on the reference RNA sequence, 17 usable non-reference RNAs and 318 usable ESTs sequences were identified. To increase the accuracy of the search, the reference RNA sequence was broken into shorter sequences (exons 1-5, 6-8, 9-11, and 12, as called in earlier publications(1)) and all search results with <90% identity were included in the analyses. The DNA Sequence Analysis Software - Sequencher 4.8 (Gene Codes Corporation; Ann Arbor, USA) was used to align and hand curate the remaining sequences, allowing for large gaps, 20 minimum overlap, and minimum match of 80%. Finally, the resultants were compared to the consensus sequences and the variations analyzed. Exons were deduced from the alignments of all transcripts (RNAs and ESTs) in order of appearance on the RNA sense orientation in SLC2A14 locus.

6.3.2 TISSUE EXPRESSION

Tissue expression was determined using mixed models of available bioinformatics information, quantitative PCR and regular PCR.

The abundance of the human mRNA in specific tissues was deduced from RNA sequencing data, determined by the Reference Transcriptome Resource (http://nhprtr.org/) (11). The abundance of reads was assessed by the magic index against the intergenic background and expression values were normalized as significant Fragments Per Kilobase Of Exon Per Million Fragments Mapped (sFPKM) (11).

Quantitative real time PCR was performed on marathon ready cDNA from human small intestine, testis, kidney, and liver (Clontech Laboratories Inc., Mountain View, CA; cat# 639326, cat#639314, cat#639305, cat#639307,respectively), on a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA), using a custom made PrimeTime® Mini qPCR Assay (MiniPrb6-FAM/ZEN/IBFQ) (Integrated DNA Technologies, Coralville, Iowa, USA), with the following specifications: sense primer GAAGAGAAATTGGAGAGGGAGTC, antisense primer GTGTGCGGCTTCATTCTTG, probe CTGGAATTGGTGGGTTCTTGGTCTCA.

PCR was performed on Marathon Ready cDNA (Clontech, Mountain View, CA, USA) from liver (cat#639307), small intestine(cat#639326), testis (cat#639314) and placenta(cat#639311) using the following primers: sense GAGATGGACAACAGACAG, antisense GACATTGGTGGTGGTCTCCTTA. Amplicons were separated by DNA-gel electrophoresis on

a 1.5% agarose gel in TAE buffer, containing ethidium bromide and imaged in a ChemiDoc gel imaging systems (Bio-Rad Laboratories, Mississauga, ON, Canada).

6.3.3 SUBCLONING OF SLC2A14/GLUT 14 ISOFORMS

Subcloning was performed using PCR techniques with Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswitch, Massachusetts), following the manufacturers guidelines. The two major testis specific transcripts(12), which encode for two protein isoforms with very high homology to GLUT3, were subcloned.

The shorter GLUT14 proteins isoforms open reading frame was amplified from cDNA clone 5297510 (plasmid: Open Biosystems catalogue # MHS1010-9204259; GENEBANK: BC060766). This major testis specific transcript skips exon eleven, creating a start codon in exon ten and encodes for the shorter N-terminus (MDNRQN) (1), which is after these initiates six amino acids in frame with the longer isoform. The following primers were utilized: sense GAGATGGACAACAGACAG, antisense GACATTGGTGGTGGTCTCCTTA. This amplicon was C-terminally tagged with the red fluorescent protein mCherry (Clontech Laboratories) using a PCR based approach of overlapping primers: sense hybrid primer for GLUT14 and mCherry: TAAGGA-GACCACCACCAATGTC-ATGGCCAGCAAAGGAGAAGA. The mCherry was amplified from plasmid pcDNA™-DEST53 Vector (Life Technologies) using the following primers: sense ATGGCTAGCAAAGGAG-AAGA, antisense GACCACCACCAATGTCTAA. The GLUT 14 amplicon and the mCherry amplicon were ligated in a third round of PCR and tagged with the "Gateway attB1 and attB2" sequences to allow transfer of the amplicon into the

Gateway® (Life Technologies) subcloning system. The final amplicon was subloned into pDONR™221 Vector and expression vector pcDNA3.2/V5-DEST (Life Technologies).

The longer GLUT14 protein isoforms open reading frame was amplified from testis marathon ready cDNA (catalogue # 639314, Clontech Laboratories, Mountain View, California) using the following primers: sense ATGGAGTTTCACAATGGTGG, antisense GACATTGGTGGTG-GTCTCCTTA. The reference sequence RNA NM_153449.2, represents the longer version of the protein, utilizing a start codon in exon 11, encoding a protein in frame with the shorter isoform, but with a distinct N-terminus of 30 amino acids (1). This amplicon was C-terminally tagged with the green fluorescent protein GFP-Cycle 3using a PCR based approach of overlapping primers: sense hybrid primer for GLUT14 and GFP: TAAGGAGACCACCACCAATGTCATGGCCAGCAAAGGAGAAGA. GFP was amplified from plasmid pReceiver-M55(a,x,y) (GeneCopoeia, Rockville, MD, USA) using the following primers: sense ATGGTGAGCAAGGGCGAGGA, antisense TCACTTGT-ACAGCTCGTCCAT. The GLUT 14 amplicon and the tGFP amplicon were ligated in a third round of PCR and tagged to transfer them into the Gateway® (Life Technologies) subcloning system. The final amplicon was subloned into pDONR™221 Vector and expression vector pcDNA3.2/V5-DEST (Life Technologies). The identity of the inserts was confirmed by sequencing. The following two plasmids are derived of this process: A) tGFP-SLC2A14-Long-pcDNA/V5-DEST; B) mCherry-SLC2A14-Short-pcDNA/V5-DEST.

6.3.4 MOLECULAR IMAGING

Chinese Hamsters Ovary cells (CHO-K1) were purchased from American Type Culture Collection (ATTC, Manassas, Virginia, USA), and maintained on 24-well tissue culture plates (Thermo Fisher Scientific, BioLite) at 37°C in DMEM/F-12 HyClone media (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). At 60-80% confluency the GLUT14 expression plasmids (mCherry-SLC2A14-Short pcDNA/V5-DEST or tGFP-SLC2A14-Long pcDNA/V5-DEST) were transfected into the cells using Effectene Transfection Reagent (Quiagen), following the manufacturer's protocols. After 24 to 72 hours, transfected cells were imaged at an inverted Axiovert 200M Fluorescence/Live cell Imaging Microscope (Carl Zeiss GMBH, Jena, Germany) using standard green and red filter sets. Images were collected with the Axiovision software package AxioVS40 v 4.5, and were processed using Zen Software (Carl Zeiss GMBH).

6.4 RESULTS

6.4.1 GENOMIC LOCUS

The revised genomic organization of the *SLC2A14* gene shows a total of twenty exons covering 103,477 nucleotides from the first Transcriptional Start Site (TSS) to the termination of the longest transcript. *SLC2A14* is transcribed from the complement strand of chromosome 12p13.31 (**Figure 6.1**). It is in tandem with the paralogue *SLC2A3* gene, which is located 3232 nucleotides upstream but only has a size of one fourth of *SLC2A14* gene (**Figure 6.1**). The locus overlaps the LOC100130582, ROS20P29, and NANOGP1 genes, which are transcribed from the opposite

strand (**Figure 6.1**). *SLC2A14* orthologues are only found in primates, whereas *SLC2A3* orthologues are found throughout the animal kingdom (Appendix I, chapter VI, Figure 1).

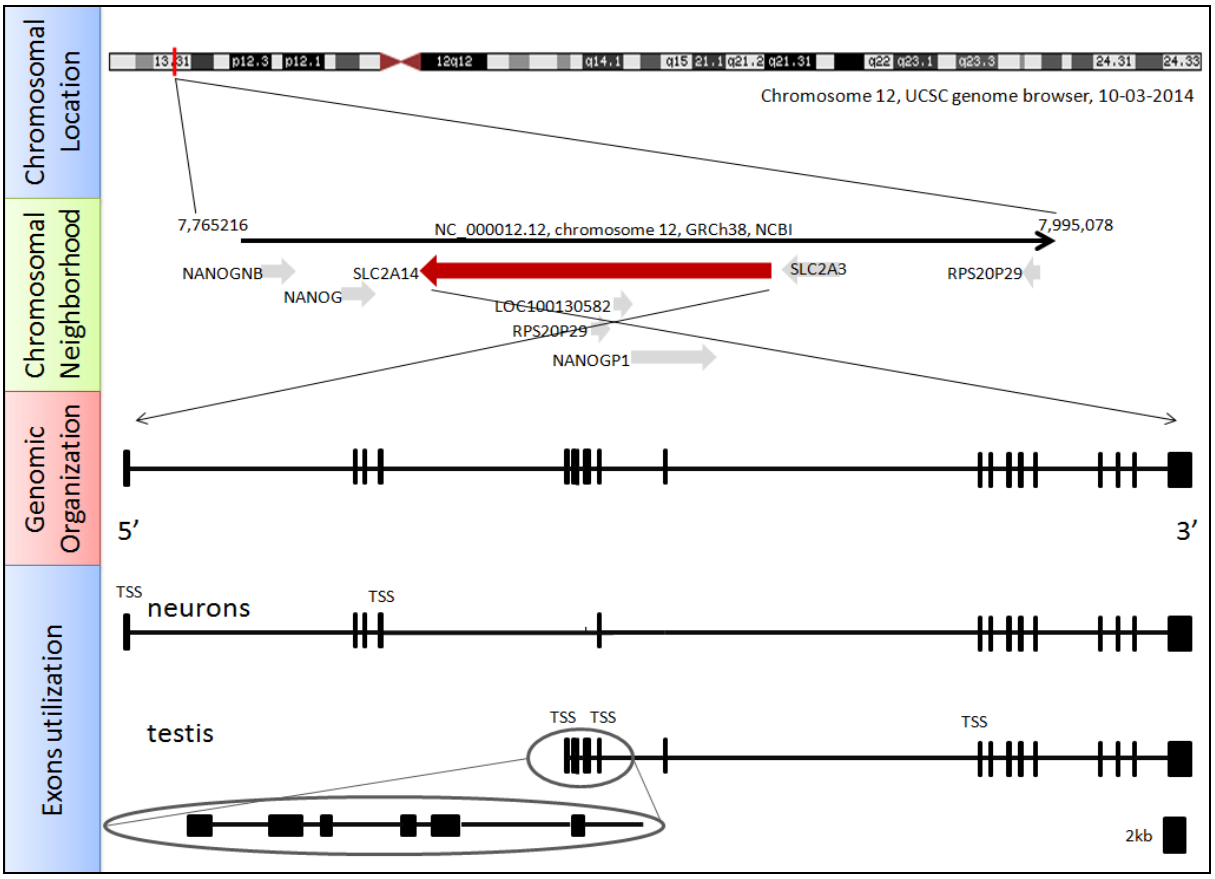


Figure 6.1: Chromosomal location, overlapping and neighboring genes, overall genomic organization and tissue specific exon utilization of the SLC2A14 gene.

6.4.2 TISSUE EXPRESSION

SLC2A14 is highly expressed in testis, but according to publicly available sources, significant levels of mRNA are also found in the colon, lung, ovaries and blood cells, brain, skeletal muscle, heart, kidney, and liver (**Figure 6.2A**). Real Time PCR concurs with these data, showing

strongest expression in testicular tissue, and lesser but significant expression in small intestine, liver and kidney (**Figure 6.2B**).

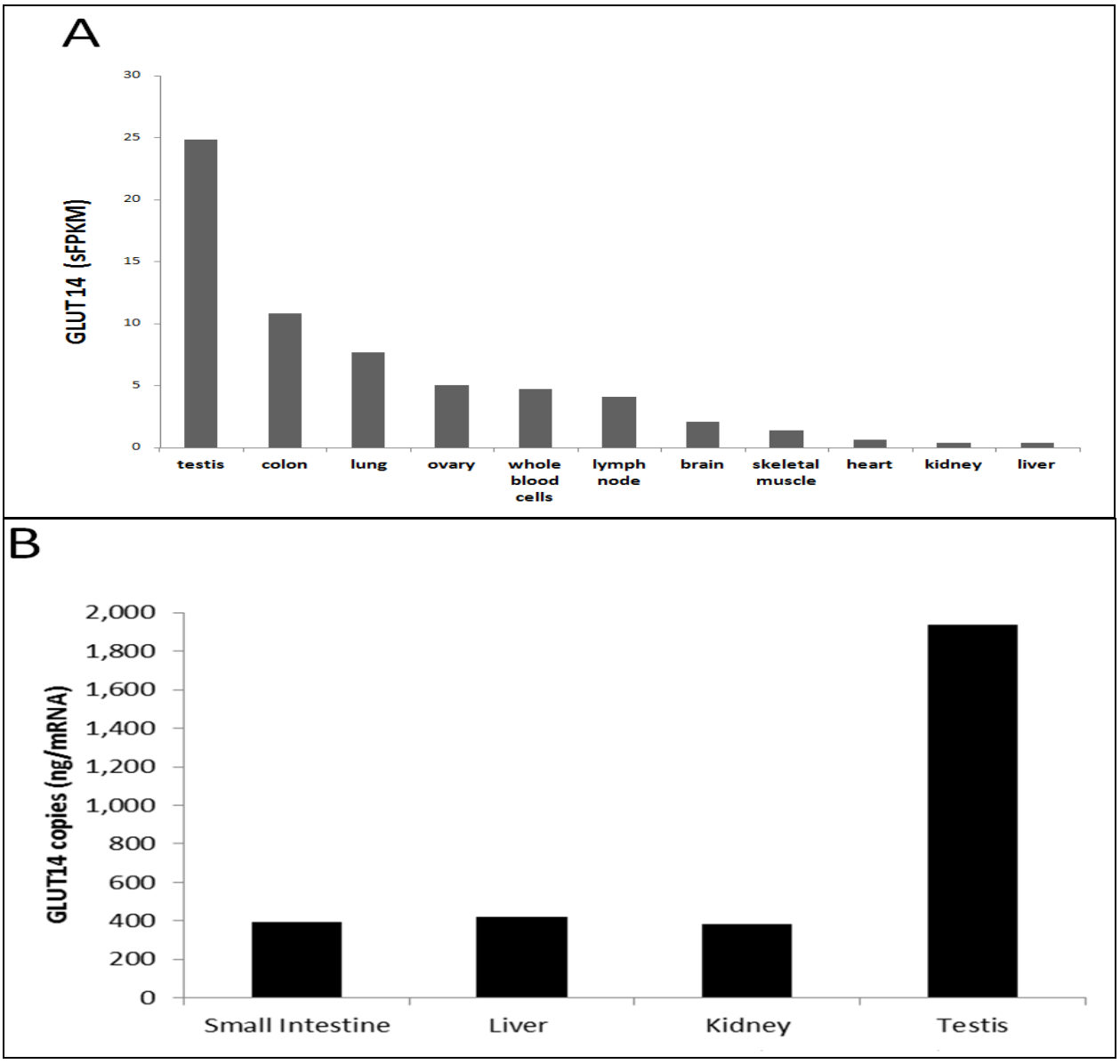


Figure 6.2: Tissues expression of SLC2A14. Panel A demonstrates the tissues of significant RNA expression as determined from publicly available RNA-sequencing datasets and expressed in significant Fragments Per Kilobase Of Exon Per Million Fragments Mapped (sFPKM) (11) (see material and methods). Panel B establishes expression in testis, small intestine, liver and kidney through quantitative Real Time PCR.

Presence in placenta is demonstrated by the fact that GLUT14 could be subcloned out of placental, liver and small intestinal cDNA (Appendix I, chapter VI, Figure 2).

6.4.3 EXON UTILIZATION AND ALTERNATIVE SPLICING

Several differentially spliced transcripts show tissue specific exon utilization, specifically in the 5' portion of *SLC2A14*.

The first four exons are exclusively utilized in brain hippocampal neuronal cells, where two distinct transcriptional start sites (TSS) in exon one and exon four exist (**Figure 6.1**). Exon five is an alternative transcriptional start site in testicular tissue (**Figure 6.1**) and is alternatively spliced at its 3' end (Appendix I, chapter VI, Figure 3). Exons five to nine are utilized in testicular tissue, but not in neuronal (**Figure 6.1**).

One third of the neuronal transcripts utilize exon ten, while all testis transcripts contain it. In addition, exon ten is a major alternative transcriptional start site for testicular transcripts. Exon eleven stands out in regard to splicing patterns, through showing tissue specific utilization as well as an intra-tissue splicing pattern. All neuronal transcripts skip exon eleven. All testis specific transcripts originating from the transcriptional start site in exon ten skip exon eleven, while it is contained in 47% of all transcripts originating from the earlier transcriptional start site in exon five (Appendix I, chapter VI, Figure 4).

Exons twelve through twenty are utilized in all tissues. Exon twelve also represents the last identifiable transcriptional start site (**Figure 6.1**), where neuronal as well as testicular transcripts originate. Exon 13 is skipped in about 3.5% of transcripts (Appendix II, chapter VI, Figure 5). The first 198 nucleotides of the last exon (exon 20) are skipped in 7% of the transcripts (Appendix I, chapter VI, Figure 6).

6.4.4 TRANSCRIPTS TERMINATION SITES

The last exon contains a maximum of 2014 nucleotides, where 3% of the transcripts support this longest extension. At least two earlier transcription termination sites are apparent, one resulting in a 1003 nucleotide exon and the other in a 733 nucleotide exon, the latter is equivalent to the termination site in the paralogue SLC2A3 gene. All of the alternative transcript termination sites do not impact on the open reading frame.

6.4.5 TRANSCRIPTS

Three major transcripts encoding for two protein isoforms exist in testicular tissue. The shortest transcript originates in exon ten and skips exon eleven, encoding for a shorter protein of 497 amino acids, originally named by Wu and Freeze GLUT14S.

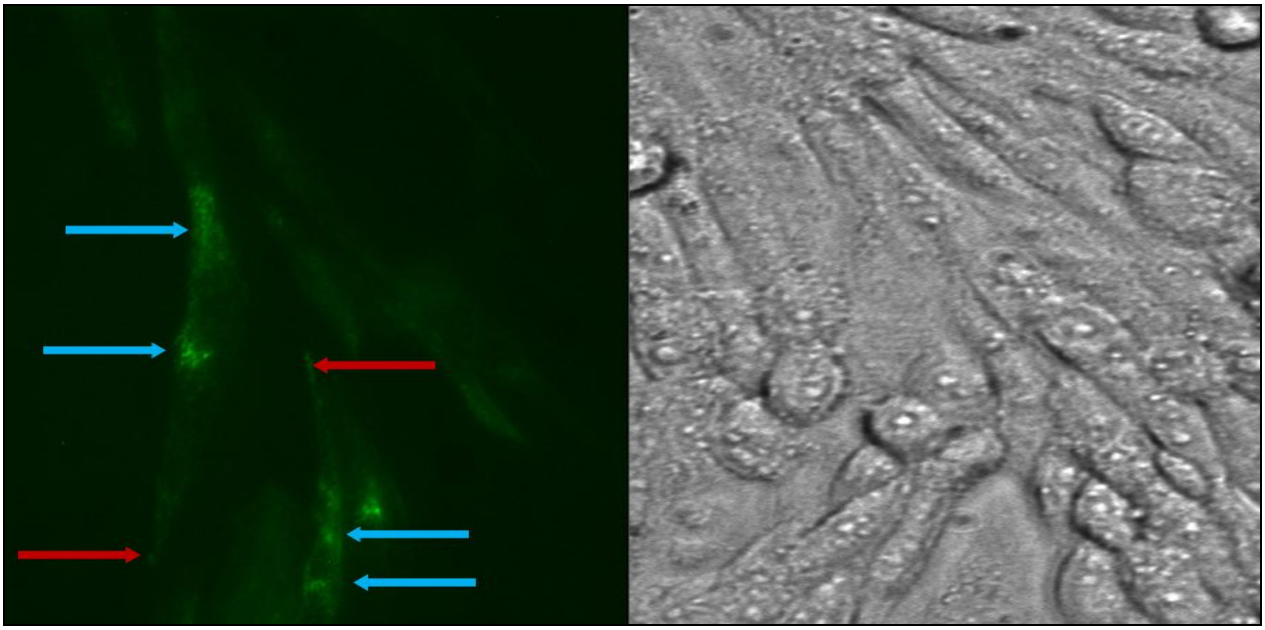
Two major transcripts originate in exon five, skip exons six to nine, and utilize exon ten. 53% of these transcripts utilize the start codon in exon ten by skipping exon eleven, encoding the GLUT14S isoform. In contrast, 47% incorporate exon 11, which disrupts the GLUT14S open

reading frame. Instead, a translation start codon located at the immediate start of exon eleven creates a novel open reading frame encoding for a longer isoform of 520 amino acid, earlier named GLUT14L (1). The two protein isoform differ in their N-terminus; GLUT14L has twenty-nine distinct amino acids, where GLUT14S has six, consistent with the isoforms described earlier (1).

6.4.6 SUBCELLULAR LOCALIZATION

Upon expression of both major fluorescently labeled GLUT14 isoforms in CHO-K1 cells a subcellular localization to the membrane can be observed. There is also a perinuclear intracellular localization, consistent with the sites of synthesis and processing on the Endoplasmic Reticulum and the Golgi Apparatus (**Figure 6.3**).

A



B

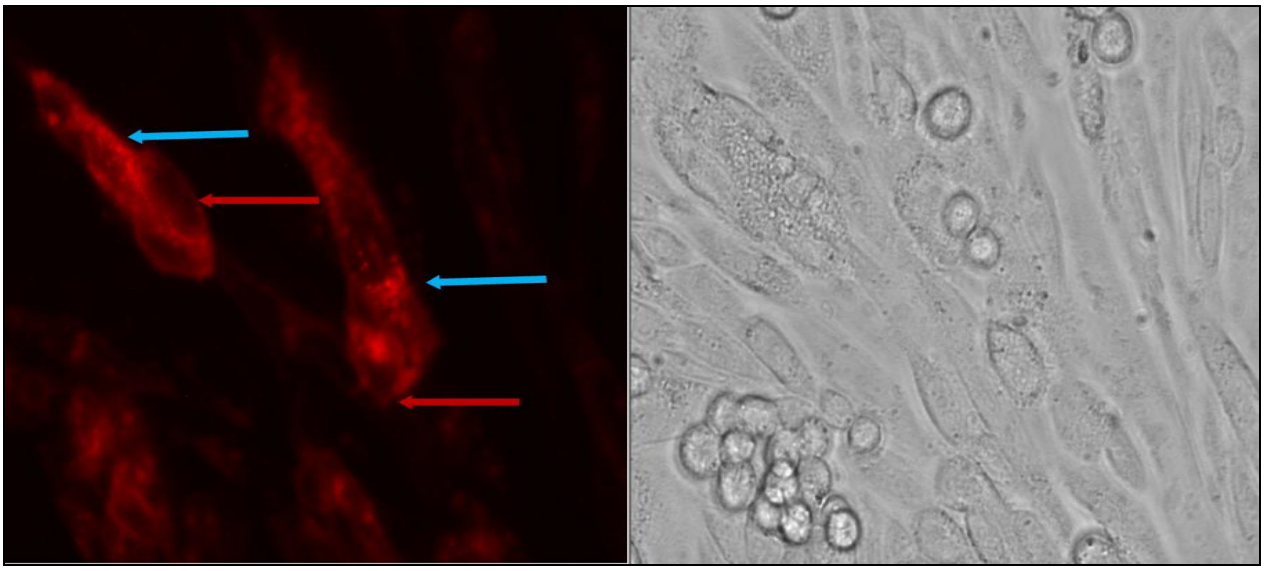


Figure 6.3: The two major testicular GLUT14 isoforms are synthesized and processed on the Endoplasmic Reticulum/Golgi Apparatus and locate to the plasmalemma membrane in CHO-K1 cells. Upon expression, fluorescent signals for the GLUT14 long (green, panel A) and the GLUT14 short isoforms (red, panel B) can be detected perinuclear at the site of synthesis and processing (endoplasmic reticulum/Golgi Apparatus, blue arrows) as well as on the plasmalemma membrane (red arrows). The fluorescent images are matched with the correspondent light images to show the cells alignment in a tight monolayer.

6.5 DISCUSSION

In expansion of prior work (1) we describe ten additional exons originating from the *SLC2A14* gene locus; therefore, according to the current analysis it contains twenty exons. We named the exon in the order of appearance, consistent with latest recommendations (hgsv.org, http://www.hgvs.org/mutnomen/refseq.html#exonnumber). Only one prior publication (1) numbered *SLC2A14* exons; however, the locus was only partially described. Wu and Freezes (1) first exon (exon 1a in the Wu and Freeze publication) is represented by our tenth exon, where their exon 1b is our eleventh exon, and all following exons correspond to their description of their gene structure. Therefore Wu and Freeze (1) did not describe the first five exons for testicular transcripts which are located 5' of exon ten. They also failed to determine the neuronal splice variants and therefore did not describe the neuron specifics exons one to four.

In expansion of the earlier record of gene expression (1), we find evidence for *SLC2A14* expression beyond testis. The expression in neuronal cell types could explain why genetic variations in *SLC2A14* are associated with Alzheimer's disease (7, 8) and why expression patterns vary in Parkinson's disease patients (6). The presented data therefore contribute to the understanding of future disease associations relating to *SLC2A14* as a putative expression quantitative trait locus (eQTL).

The current analysis revealed distinct tissue specific exon utilization, which could be determined for neuronal tissues and testis. The existence of five distinct transcriptional start sites with distinct promoter regions might be the hallmark of an ongoing evolution in the *SLC2A14* gene, which was only recently duplicated during primate evolution. The specific functional differences

between the protein isoform encoded by these splice variants remain to be determined. However, the major testicular protein isoforms are likely to be solute carriers on the plasmalemma membrane, similar to the highly conserved paralogue GLUT3 (1).

The existence of three transcriptional termination sites could also indicate an ongoing evolutionary process, which might distinguish GLUT14 from its paralogue GLUT3. Alternative termination in the 3' untranslated region of genes affects the scaffolding of the proteins, resulting in differential intracellular targeting (13). Although we have proven plasmalemma localization of the protein, we had used plasmids carrying the open reading frame only. In future studies it needs to be investigated how the 3' untranslated regions of the *SLC2A14* transcript determine the intracellular location of the GLUT14 isoforms or how these distinguish it from GLUT3.

In conclusion, we update the knowledge about the *SLC2A14* genomic locus, identifying ten additional exons, expression beyond testicular tissue, and tissue specific exon utilization. The major protein isoforms locate to the plasmalemma membrane, indicating a function as solute carrier.

6.6 REFERENCES

1. Wu, X.H., and Freeze, H.H. 2002. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80:553-557.

2. Cura, A.J., and Carruthers, A. 2012. Role of monosaccharide transport proteins in carbohydrate assimilation, distribution, metabolism, and homeostasis. *Comprehensive Physiology* 2:863-914.

3. Januchowski, R., Zawierucha, P., Andrzejewska, M., Ruciński, M., and Zabel, M. 2013. Microarray-based detection and expression analysis of ABC and SLC transporters in drug-resistant ovarian cancer cell lines. *Biomedicine and Pharmacotherapy* 67:240-245.

4. Januchowski, R., Zawierucha, P., Ruciński, M., Andrzejewska, M., Wojtowicz, K., Nowicki, M., and Zabel, M. 2014. Drug transporter expression profiling in chemoresistant variants of the A2780 ovarian cancer cell line. *Biomedicine and Pharmacotherapy* 68:447-453.

5. Taylor, K.H., Pena-Hernandez, K.E., Davis, J.W., Arthur, G.L., Duff, D.J., Shi, H., Rahmatpanah, F.B., Sjahputera, O., and Caldwell, C.W. 2007. Large-Scale CpG Methylation Analysis Identifies Novel Candidate Genes and Reveals Methylation Hotspots in Acute Lymphoblastic Leukemia. *Cancer Research* 67:2617-2625.

6. Infante, J., Prieto, C., Sierra, M., Sanchez-Juan, P., Gonzalez-Aramburu, I., Sanchez-Quintana, C., Berciano, J., Combarros, O., and Sainz, J. 2015. Identification of candidate genes for Parkinson's disease through blood transcriptome analysis in LRRK2-G2019S carriers, idiopathic cases, and controls. *Neurobiol Aging* 36:1105-1109.

7. Shulman, J.M., Chipendo, P., Chibnik, L.B., Aubin, C., Tran, D., Keenan, B.T., Kramer, P.L., Schneider, J.A., Bennett, D.A., Feany, M.B., et al. 2011. Functional Screening of Alzheimer Pathology Genome-wide Association Signals in Drosophila. *The American Journal of Human Genetics* 88:232-238.

8. Wang, W., Yu, J.T., Zhang, W., Cui, W.Z., Wu, Z.C., Zhang, Q., and Tan, L. 2012. Genetic Association of SLC2A14 Polymorphism with Alzheimer's Disease in a Han Chinese Population. *Journal of Molecular Neuroscience* 47:481-484.

9. Veal, C.D., Reekie, K.E., Lorentzen, J.C., Gregersen, P.K., Padyukov, L., and Brookes, A.J. 2014. A 129-kb Deletion on Chromosome 12 Confers Substantial Protection Against Rheumatoid Arthritis, Implicating the Gene SLC2A3. *Human Mutation* 35:248-256.

10. Nag, A., Venturini, C., Hysi, P.G., Arno, M., Aldecoa-Otalora Astarloa, E., Macgregor, S., Hewitt, A.W., Young, T.L., Mitchell, P., Viswanathan, A.C., et al. 2013. Copy number variation at chromosome 5q21.2 is associated with intraocular pressure. *Investigative ophthalmology & visual science* 54:3607-3612.

11. Pipes, L., Li, S., Bozinoski, M., Palermo, R., Peng, X., Blood, P., Kelly, S., Weiss, J.M., Thierry-Mieg, J., Thierry-Mieg, D., et al. 2013. The non-human primate reference transcriptome resource (NHPRTR) for comparative functional genomics. *Nucleic Acids Res* 41:D906-914.

12. Wu, X., and Freeze, H.H. 2002. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80:553-557.

13. Berkovits, B.D., and Mayr, C. 2015. Alternative 3[prime] UTRs act as scaffolds to regulate membrane protein localization. *Nature* advance online publication.

TRANSITION STATEMENT 4

Focusing on *SLC2A14* genomics, Chapter VI demonstrated expression of *SLC2A14* (GLUT 14) in the intestinal tract. Moreover, Chapter V reported an association for the ASC transporter gene *SLC23A1* with the IBD, strengthens our overall hypothesis that localized intestinal antioxidants imbalances contribute to the aetiology of IBD. During inflammation extracellular ASC is oxidized to DHA which then transport back, through GLUT transporters, into the cell. Upon entry into the cell DHA is immediately reduced back to ASC and this mechanism maintains intracellular redox balance. Besides, in many cell types including neutrophils, the rate of DHA transport through GLUTs exceeds the transport of ASC by SLC23s, leading to increase intra cellular ASC. Consequently, in the condition of inflammation or for specific cell types, the role of GLUTs transporters, including GLUT 14, may be even more important to sustain intra cellular ASC levels, compared to SLC23s transporters. Therefore we determined the genetic associations of *SLC2A14* with IBD.

In the following chapter, we characterized the GLUT 14 protein as a transporter for DHA and also determined strong associations between variations in the *SLC2A14* (GLU14) gene and IBD. This is the first evidence for the GLUT14 function as well as its genetic associations with IBD.

CHAPTER 7

MANUSCRIPT 5

This manuscript is submitted to the American Journal of Human Genetics

THE NOVEL GLUCOSE/DEHYDROASCORBATE TRANSPORTER SLC2A14 (GLUT14) IS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

Mandana Amir Shaghaghi [1], Hongbin Tu[2], Charles N. Bernstein[3,4], Hani El-Gabalawy[4], Garry Crow[5], Mark Levine[2], Peter Eck[1]

Afflications: [1]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada, R3T 2N2.[2] Molecular and Clinical Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA. [3]University of Manitoba IBD Clinical and Research Centre. [4]Department of Internal Medicine, University of Manitoba, Winnipeg, Canada. [5]Department of Animal Sciences, University of Manitoba, Winnipeg, Canada

7.1 ABSTRACT

Oxidative stress is implicated in the initiation and progression of inflammatory bowel disease (IBD) which includes Crohn's disease (CD) and ulcerative colitis (UC). Dehydroascorbic acid (DHA) is imported into cells via glucose transporters (GLUTs) and converted to the active antioxidant vitamin C. To determine whether orphan GLUT 14 has a DHA transporter function and to determine if its genetic variation is associated with IBD. The population based Manitoba IBD Cohort Study, initiated in 2002. Participants were at least 18 yr (18-80 yr) of age, diagnosed within the previous 7 yr. The identification and extent of IBD was determined based on surgical, endoscopic, radiologic, and histologic data. Caucasians with CD (n=162), UC (n=149), and ethnically matched controls (n=142). Controls were drawn from the general population, included individuals with no personal or first-degree relatives with chronic immune diseases. Genomic DNA samples from participants were genotyped for eight *GLUT 14* polymorphisms. Logistic regression was used to determine the case-control association of genotypes for each polymorphism with CD or UC. Genotype-phenotype associations were tested by multinomial logistic regression. Radiolabeled DHA transport was determined in *Xenopus laevis* oocytes injected with cRNA of the two major GLUT14 isoforms. GLUT14 mediates DHA transmembrane transport. Variant rs2889504-A was associated with UC and CD (OR: 3.60, 95% CI: 1.95, 6.64; OR: 4.68, 95% CI: 2.78, 8.50, respectively). The rs10846086-G allele was associated with an approximated 3-fold increased risk of both UC and CD (OR: 2.91, 95% CI: 1.49, 5.68; OR: 3.00, 95% CI: 1.55, 5.78, respectively). Variant rs12815313-T was associated with increased susceptibility to CD (OR: 2.12, 95% CI: 1.33, 3.36). These findings strengthen the evidence that a local mucosal imbalance in the major dietary water soluble antioxidant, vitamin C, is associated with IBD. This may have implications for nutritional treatments for

patients carrying the disease-associated genotypes. Further studies are needed to elucidate specific IBD prevention strategies.

7.2 INTRODUCTION

Cellular oxidative events continuously generate reactive oxygen species (ROS) which can harm biological macromolecules (1). ROS play a key role in the pathophysiology of tissue injury in inflammatory bowel disease (IBD), mainly through the initiation and continuation of the inflammatory cascade which begins with massive infiltration of leukocytes into the intestinal wall(2). ROS level in colonic biopsy specimens of IBD patients is higher than the normal mucosa(3-6) and these elevated ROS levels cause oxidative damage (7-9), further recruitment of inflammatory cells, and thus compromising tissue integrity and function (2, 10). ROS also enhance intestinal inflammation through the expression of a variety of immune molecules, promoting neutrophil adherence, and increasing mucosal and vascular permeability (11-13).

Vitamin C is an essential dietary antioxidant known to terminate or retard the oxidative damage and protect tissues (14) through decreasing oxidative stress in IBD (15). Vitamin C directly scavenges intracellular ROS to protect from oxidative cell death(16-18), prevents DNA mutation (19), and inhibits FAS-induced apoptosis (20). Human cells transport ascorbate (ASC), the active form of vitamin C, through sodium dependent ascorbic acid transporters (21, 22); they also transport DHA, the oxidized form of vitamin C via facilitative diffusion through glucose transporters (GLUTs)(23-26).

During the inflammatory oxidative burst activated cells secrete ROS that oxidize extracellular ASC to DHA(27), which is transported into the cell and immediately reduced back to ASC(28, 29). This phenomenon leads to increased accumulation of vitamin C by the activated cells(27) and by any cell present in the immediate vicinity, providing those cells with increased resistance to oxidative stress and cellular protection(28, 30).

The orphan *SLC2A14* gene (solute carrier family 2 member 14) is exclusively found in primates and encodes GLUT14, a putative facilitated glucose transporter, which has major expression in testis, but is also expressed in the intestinal tract (Amir Shaghaghi et al. 2015, reference to be created still). Based on high homology to GLUT 3(31), which is a known high capacity DHA transporter(32), we hypothesize that GLUT 14 is also a DHA transporter and genetic variation could deregulate its function and results in intracellular redox imbalance and thus increased risk of IBD. This notion is enforced by the fact that recently genetic variation of *SLC23A1*, encoding a major intestinal ascorbate transporter, was associated to Crohn's disease(33).

In this manuscript we characterized the DHA transport function of GLUT 14 and genetic associations with IBD.

7.3 METHODS

7.3.1 DESCRIPTION OF THE COHORT AND PARTICIPANTS

The Manitoba IBD Cohort Study, initiated in 2002 included 388 persons drawn from a population based registry. Participants were required to be at least 18 yr (18-80 yr) of age and

diagnosed within the previous 7 yr. The diagnosis and extent of IBD was determined based on surgical, endoscopic, radiologic, and histologic data and participating individuals were phenotyped using the Montreal classification (34). Controls, drawn from the general population, included healthy individuals with no personal or first-degree relatives with chronic immune diseases. Of the 388 persons in the Cohort 311 IBD patients (162 CD and 149 UC patients) were Caucasian and 142 age- and sex-matched healthy Caucasian controls were included in the study. More details on the study design and creation of this study population are previously provided (35). The Manitoba IBD Cohort Study was approved by the Research Ethics Board of the University of Manitoba.

7.3.2 TRANSPORT STUDIES

As previously described elsewhere, the two major GLUT14 isoforms were subcloned (Amir Shaghaghi et al. 2015, reference to be created still). Radiolabelled [^{14}C]DHA was prepared from crystalline [^{14}C]Asc (6.6 mCi/mmol, PerkinElmer Life Sciences)(26). Total conversion of [^{14}C]Asc to DHA was confirmed using HPLC with electro-coulometric detection(36). Previously described *Xenopus laevis* oocyte isolation and injection techniques(37), were used to express the GLUT14 isoforms.

Transport of [^{14}C]DHA was determined using groups of 10–20 oocytes in OR-2 buffer containing 250 μmol/l of [^{14}C]DHA (0.6–5.5 μCi/ml) for 10, 30, and 60 min at 23°C. Individual oocytes were dissolved in 500 μl of 10% SDS, and internalized radioactivity was determined using scintillation spectrometry. DHA transport was analyzed and plotted using Microsoft Excel,

Student's *t* test was used to determined statistical differences. Data are expressed as the arithmetic mean ± S.D. of 10–20 oocytes analyzed at each data point.

7.3.3 SINGLE NUCLEOTIDE POLYMORPHISM SELECTION, GENOTYPING METHODS, AND ASSOCIATION ANALYSES

A haplotype-based tag-SNP approach was implemented(38) using the eight tagging SNPs: rs10846086, rs12815313, rs2889504, rs10845990, rs11612319, rs7132415, rs2376904, and rs73007730. These captured all the common variation (MLF >5%) in *SLC2A14* (Appendix I, chapter VI, Table 1).

Genomic DNA was isolated from peripheral white blood cells by absorption onto QIamp silics-gel following QAIGEN protease digestion (QIAGEN, Mississauga, Canada). Subsequent to column elution, the purity and concentration of DNA was determined by UV spectroscopy (BioRad, Mississauga, Canada).

Genotyping was performed for all subjects for the eight tag-SNPs using TaqMan Real-Time polymerase chain reaction assays (Applied Biosystems, Grand Island, New York, USA). The genotype concordance rate was 100% in duplicate samples.

All data were analyzed using SAS version 9.2 (SAS Institute Inc, Cary, NC); $P < .05$ was considered statistically significant. The association of genotypes in each of the eight SNPs with CD or UC risks was examined by logistic regression to estimate ORs and 95% CIs, using a

genotypic model with three levels (two homozygotes and one heterozygote). Overdominance was tested only in those situations where the heterozygote was not intermediate in effect between the two homozygotes. The genotype-phenotype association for individuals with CD and UC was determined using Multinomial logistic regression. Linkage disequilibrium and haplotype blocks were performed using Haploview 4.2 software (Broad Institute)(39), the default method (40).

7.4 RESULTS

7.4.1 *SLC2A14* SINGLE NUCLEOTIDE POLYMORPHISSM INDEPENDENTLY ASSOCIATE WITH INFLAMMATORY BOWEL DISEASE

Baseline characteristics of participants are presented in the (Appendix I, chapter VI, Table 2). Genetic variations in SNPs rs10846086, rs2889504 associated with UC and CD, while rs12815313 associated with CD only (**Table 7.1)**. No linkage was observed for the eight tag-SNPs in the *SLC2A14* gene (**Figure 7.1**), and the pattern of inheritance for the three SNPs associated to any disease phenotype differed significantly (**Table 7.1**).

First, the susceptibility for CD was elevated in individuals carrying the SNP rs12815313-T allele (OR: 2.12, **Table 7.1**), where impact sizes are equal for homozygotes and CT-heterozygotes (OR: 2.04 for CT, and OR: 2.41 for TT, **Table 7.1**). However, no association was observed for UC **(Table 7.1)**.

Second, the rs10846086-G allele elevated risks for UC and CD (ORs: 2.91, 3.00, respectively, **Table 7.1**). An additive allele dosage effect was demonstrated, where highest susceptibility for

UC and CD was observed for rs10846086-GG homozygotes (ORs: 4.30, 4.20, respectively, **Table 7.1**), while the impact size was about halved for rs10846086-AG heterozygotes (ORs: 2.14, 2.33, respectively, **Table 7.1**).

Third, the presence of the SNP rs2889504-T allele increased susceptibility to UC and CD (ORs: 3.60, 4.68, respectively, **Table 7.1**). Rs2889504-GT heterozygotes exhibited the highest risk for UC and CD (ORs: 5.36; 6.49, respectively, **Table 7.1**) compared to T-allele homozygotes (ORs: 2.37, 3.41, respectively, **Table 7.1**). However, over-dominance could not be proven for the impact sizes observed for the rs2889504-GT genotypes.

For all SNPs associated to CD or UC, no significant correlations were found to specific sub-phenotypes of CD and UC (data not shown).

Table 7.1: Genetic associations of SNP in the *SLC2A14* gene to Ulcerative colitis and Crohn´s disease [1]

	Controls n=142	**Ulcerative colitis n=149**	**OR (95% CI)[2] UC vs. Control**	**Crohn´s disease n=162**	**OR (95% CI)[2] CD vs. Control**
	No. (%)	No. (%)		No. (%)	
rs12815313					
CC	73 (51.4)	59 (39.6)	REF	54 (33.4)	REF
CT	55 (38.7)	71 (47.6)	1.59 (0.98-2.61)	83 (51.2)	**2.04 (1.25-3.33)**
TT	14 (9.9)	19 (12.7)	1.68 (0.78-3.63)	25 (15.4)	**2.41 (1.15-5.07)**
T-carrier	69 (48.59)	90 (60.4)	1.61 (1.01-2.57)	108 (66.7)	2.12 (1.33-3.36)
Dominance			0.81 (0.48-1.37)		0.76 (0.46-1.26)
rs10845990					
TT	34 (23.9)	29 (19.5)	REF	32 (19.7)	REF
GT	68 (47.9)	64 (42.9)	1.10 (0.60-2.01)	76 (46.9)	1.19 (0.66-2.13)
GG	40 (28.2)	56 (37.6)	1.64 (0.86-3.11)	54(33.4)	1.43 (0.76-2.70)
G-carrier	108 (76.1)	120 (80.5)	1.30 (0.74-2.28)	130 (80.25)	1.28 (0.74-2.21)
Dominance			1.16 (0.73-1.85)		1.01 (0.64-1.59)
rs11612319					
GG	66 (46.5)	65(43.6)	REF	79(48.8)	REF
GA	66 (46.5)	69 (46.3)	1.06 (0.66-1.72)	65 (40.1)	0.82 (0.51-1.32)
AA	10 (7.0)	15(10.1)	1.52 (0.64-3.64)	18(11.1)	1.50 (0.65-3.48)
A-carrier	79 (53.5)	84 (56.4)	1.12 (0.71-1.78)	83 (51.2)	0.91 (0.58-1.43)
Dominance			1.16 (0.67-2.02)		1.49 (0.87-2.56)
rs7132415					
GG	37 (26.1)	36(24.2)	REF	31(19.1)	REF
GT	70 (49.3)	76 (51.0)	1.12 (0.64-1.96)	72 (44.5)	1.23 (0.69-2.19)
TT	35 (24.6)	37(24.8)	1.09 (0.57-2.08)	59(36.4)	2.01 (1.07-3.79)
T-carrier	105 (73.9)	113 (75.8)	1.11 (0.65-1.88)	131 (80.9)	1.49 (0.87-2.56)
Dominance			0.93 (0.59-1.48)		1.15 (0.73-1.82)
rs10846086					
AA	128 (90.1)	113 (75.8)	REF	122 (75.4)	REF
AG	9 (6.3)	17(11.4)	**2.14 (0.92-4.99)**	20 (12.3)	**2.33 (1.02-5.32)**

GG	5 (3.5)	19 (12.8)	**4.30 (1.56-11.90)**	20 (12.3)	**4.20 (1.53-11.53)**
G-carrier	14 (9.9)	36 (24.2)	**2.91 (1.49-5.68)**	40 (24.7)	**3.00 (1.55-5.78)**
Dominance			0.97 (0.37-2.52)		0.88 (0.34-2.24)
rs2376904					
GG	93 (65.5)	83 (55.7)	REF	113 (69.8)	REF
GA	43 (30.3)	58 (38.9)	1.51 (0.92-2.47)	37 (22.8)	0.70 (0.42-1.19)
AA	6 (4.2)	8 (5.4)	1.49 (0.50-4.48)	12 (7.4)	1.65 (0.59-4.55)
A-carrier	49 (34.5)	66 (44.3)	1.51 (0.94-2.42)	49 (30.2)	0.82 (0.51-1.33)
Dominance			0.81 (0.41-1.59)		1.81 (0.92-3.55)
rs7300773					
TT	50 (35.2)	47 (31.5)	REF	57 (35.2)	REF
CT	74 (52.1)	74 (49.7)	1.06 (0.64-1.78)	76 (46.9)	0.90 (0.54-1.48)
CC	18 (12.7)	28 (18.8)	1.65 (0.81-3.37)	29 (17.9)	1.41 (0.70-2.85)
CT+CC	92 (64.8)	102 (68.5)	1.18 (0.72-1.92)	105 (64.8)	1.00 (0.62-1.60)
Dominance			1.21 (0.75-1.95)		1.31 (0.82-2.12)
rs2889504					
GG	125(88.0)	100 (67.1)	REF	99 (61.1)	REF
GT	7(4.9)	30 (20.1)	**5.36 (2.26-12.71)**	36 (22.2)	**6.49 (2.77-15.21)**
TT	10 (7.1)	19 (12.8)	**2.37 (1.06-5.34)**	27 (16.7)	**3.41 (1.57-7.38)**
T-carrier	17 (12.0)	49 (32.9)	**3.60 (1.95-6.64)**	63 (38.9)	**4.68 (2.78-8.50)**
Dominance			**0.29 (0.11-0.72)**		**0.28 (0.12-0.70)**

Note: IBD, Inflammatory bowel disease; OR, Odd ratio

[1]Per-allele effects were derived from binary logistic regression.

[2]ORs were adjusted for age and sex.

***** marks significant differences in the ORs to the reference SNP

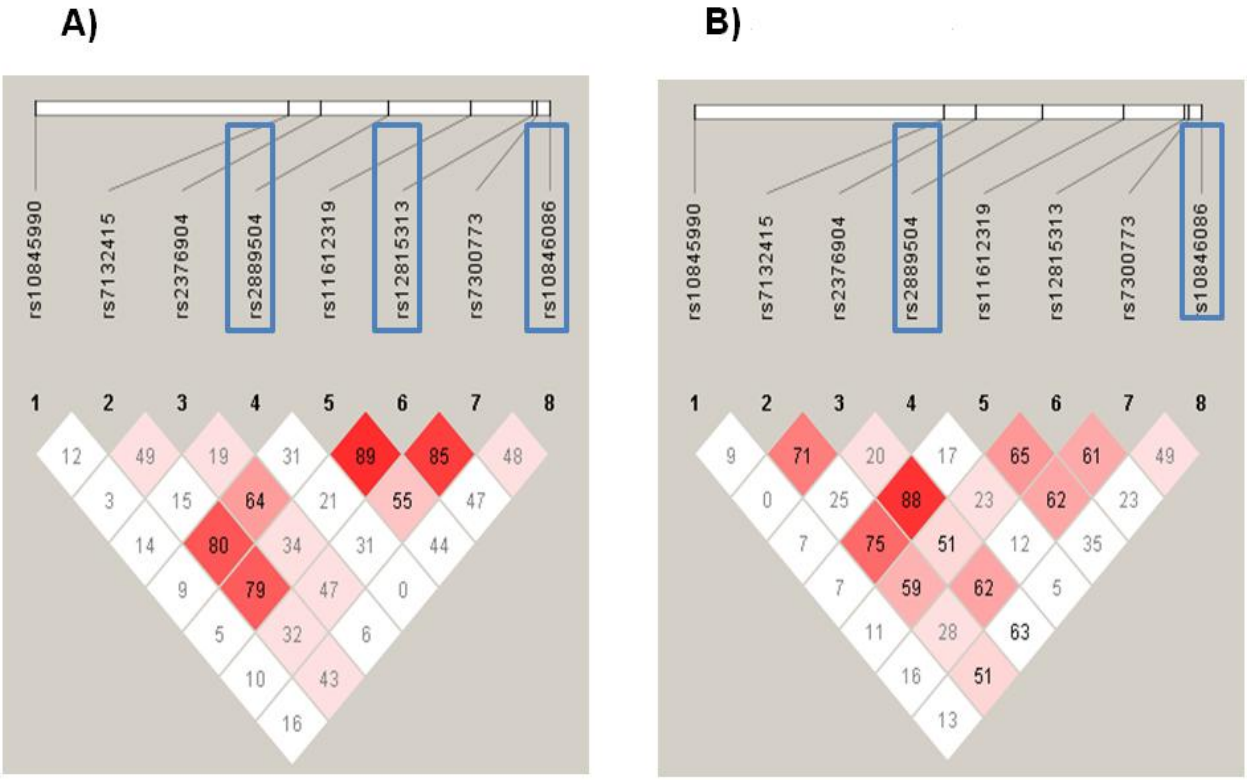


Figure 7.1: SNP are not in Genetic Linkage across the SLC2A14 locus in individuals with Crohn's disease (A) and ulcerative colitis (B). The degree of linkage (represented in the triangles) is given as a percentage, and the color red indicates a higher degree of linkage. SNPs with significant associations to any form of IBD are marked with blue rectangles. This specifically demonstrates that the SNPs associated to any form of IBD are not in genetic linkage.

7.4.3 GLUT14 MEDIATES CELLULAR DEHYDROASCORBIC ACID UPTAKE

Upon expression in *Xenopus laevis* oocytes the two major GLUT14 isoforms mediated uptake of radiolabeled dehydroascorbic acid in a time dependent manner (**Figure 7.2**).

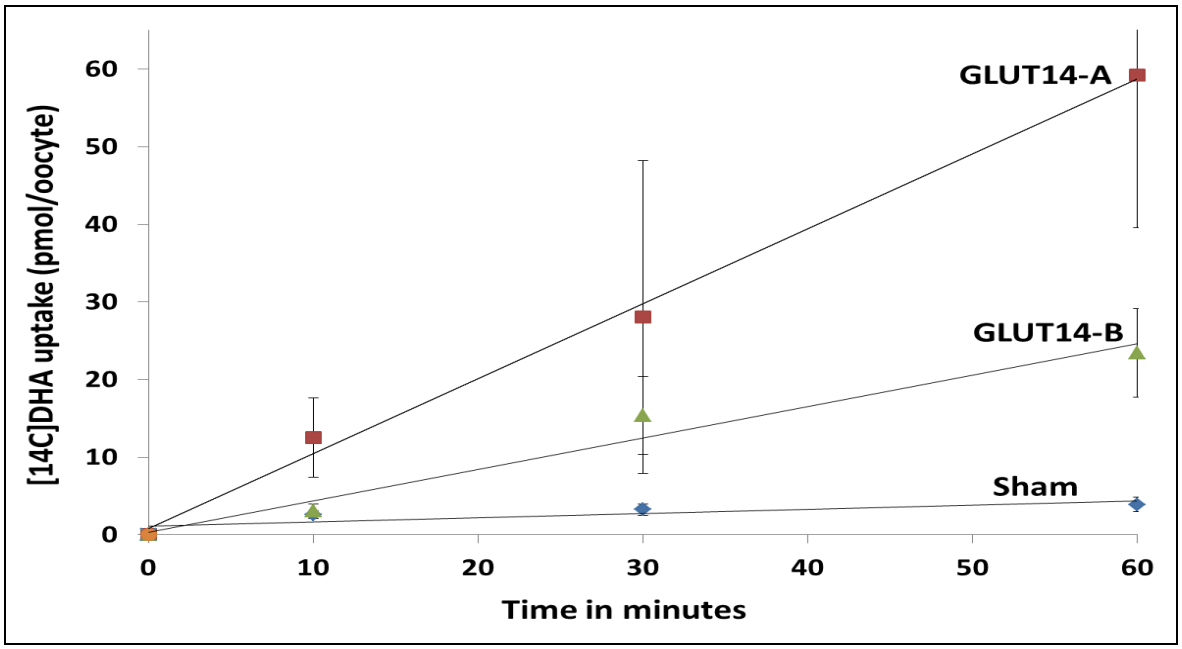


Figure 7.2: Two GLUT 14 isoforms mediate dehydroascorbic acid (DHA) uptake. Xenopus laevis oocytes express the two major GLUT 14 isoforms, exhibiting uptake of radiolabeled dehydroascorbic acid. The graph depicts one out of three independent experiments, as described in material and methods, which all yielded similar results.

7.5 COMMENTS

The two major GLUT14 isoforms mediate the uptake of dehydroascorbate. Three SNPs in the *SLC2A14* gene associate with the risk for CD and UC in a representative Caucasian cohort.

These findings strengthen the evidence that a local mucosal imbalance in the major dietary water soluble antioxidant, vitamin C, is associated with IBD. Low mucosa tissue levels (35-73%) and also low plasma concentrations of vitamin C have been reported in individuals with IBD (41-44). Since genetic variations in two intestinal vitamin C transporters genes, *SLC23A1*(33) and *SLC2A14*, are associated with IBD, we speculate that a localized vitamin C deficiency due to a lack of adequate transmembrane transport might be contributing to these diseases. We further

speculate that the decreased vitamin C content in the inflamed mucosa is caused by decreased transmembrane transport, rather than increased consumption through ROS scavenging.

While the presented data do not define underlying mechanisms, two scenarios appear logical. First, variations in GLUT14 could impact on the capacity to provide intracellular ASC into enterocytes via the bystander effect (28) in conditions of oxidative burst. As a consequence, the intestinal barrier function would be compromised, leading to increased bacterial invasion and more severe or sustained inflammation. Second, the functioning of the immune cells themselves could be disturbed due to disturbed DHA transport, enhancing the severity of the inflammation(27, 45, 46).

We conducted our studies in a well phenotyped case-control cohort of moderate size, which had replicated major genetic associations with IBD identified by GWAS (47). Therefore, these results from this well phenotyped cohort of moderate size are noteworthy, but nonetheless should be reproduced in other cohorts.

If the presented findings are validated in additional large cohorts and biological studies, targeted genotype specific IBD prevention strategies could be derived(33).

7.6 ACKNOWLEDGMENTS

The authors appreciatively recognize the participants of the Manitoba IBD Cohort Study. Dr. Bernstein has consulted for Abbott Canada, Abbvie Canada, Bristol Myers Squibb, Janssen

Canada, Forest Canada, Takeda Canada, and Vertex Pharmaceuticals and has received research grants from Abbott Canada and Abbvie Canada and an education grant from Aptalis. PE, MAS, HT, HE-G, GC, and ML had no conflicts of interest.

7.7 REFERENCES

1. Halliwell, B. 1989. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br J Exp Pathol* 70:737-757.

2. Keshavarzian, A., Banan, A., Farhadi, A., Komanduri, S., Mutlu, E., Zhang, Y., and Fields, J.Z. 2003. Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease. *Gut* 52:720-728.

3. Keshavarzian, A., Sedghi, S., Kanofsky, J., List, T., Robinson, C., Ibrahim, C., and Winship, D. 1992. Excessive production of reactive oxygen metabolites by inflamed colon: analysis by chemiluminescence probe. *Gastroenterology* 103:177-185.

4. Sedghi, S., Fields, J.Z., Klamut, M., Urban, G., Durkin, M., Winship, D., Fretland, D., Olyaee, M., and Keshavarzian, A. 1993. Increased production of luminol enhanced chemiluminescence by the inflamed colonic mucosa in patients with ulcerative colitis. *Gut* 34:1191-1197.

5. Lih-Brody, L., Powell, S.R., Collier, K.P., Reddy, G.M., Cerchia, R., Kahn, E., Weissman, G.S., Katz, S., Floyd, R.A., McKinley, M.J., et al. 1996. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 41:2078-2086.

6. Simmonds, N.J., Allen, R.E., Stevens, T.R., Van Someren, R.N., Blake, D.R., and Rampton, D.S. 1992. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* 103:186-196.

7. Gaginella, T.S., Kachur, J.F., Tamai, H., and Keshavarzian, A. 1995. Reactive oxygen and nitrogen metabolites as mediators of secretory diarrhea. *Gastroenterology* 109:2019-2028.

8. McKenzie, S.J., Baker, M.S., Buffinton, G.D., and Doe, W.F. 1996. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest* 98:136-141.

9. Kruidenier, L., and Verspaget, H.W. 1998. Antioxidants and mucosa protectives: realistic therapeutic options in inflammatory bowel disease? *Mediators Inflamm* 7:157-162.

10. Harris, M.L., Schiller, H.J., Reilly, P.M., Donowitz, M., Grisham, M.B., and Bulkley, G.B. 1992. Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon? *Pharmacol Ther* 53:375-408.

11. Kruidenier, L., and Verspaget, H.W. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 16:1997-2015.

12. Siemankowski, L.M., Morreale, J., and Briehl, M.M. 1999. Antioxidant defenses in the TNF-treated MCF-7 cells: selective increase in MnSOD. *Free Radic Biol Med* 26:919-924.

13. Grisham, M.B. 1994. Oxidants and free radicals in inflammatory bowel disease. *Lancet* 344:859-861.

14. Frei, B. 1999. On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. *Proc Soc Exp Biol Med* 222:196-204.

15. Aghdassi, E., Wendland, B.E., Steinhart, A.H., Wolman, S.L., Jeejeebhoy, K., and Allard, J.P. 2003. Antioxidant vitamin supplementation in Crohn's disease decreases oxidative stress. a randomized controlled trial. *Am J Gastroenterol* 98:348-353.

16. Garcia, A., Morales, P., Arranz, N., Delgado, M.E., Rafter, J., and Haza, A.I. 2009. Antiapoptotic effects of dietary antioxidants towards N-nitrosopiperidine and N-nitrosodibutylamine-induced apoptosis in HL-60 and HepG2 cells. *J Appl Toxicol* 29:403-413.

17. Witenberg, B., Kalir, H.H., Raviv, Z., Kletter, Y., Kravtsov, V., and Fabian, I. 1999. Inhibition by ascorbic acid of apoptosis induced by oxidative stress in HL-60 myeloid leukemia cells. *Biochem Pharmacol* 57:823-832.

18. Guaiquil, V.H., Vera, J.C., and Golde, D.W. 2001. Mechanism of vitamin C inhibition of cell death induced by oxidative stress in glutathione-depleted HL-60 cells. *J Biol Chem* 276:40955-40961.

19. Lutsenko, E.A., Carcamo, J.M., and Golde, D.W. 2002. Vitamin C prevents DNA mutation induced by oxidative stress. *J Biol Chem* 277:16895-16899.

20. Perez-Cruz, I., Carcamo, J.M., and Golde, D.W. 2003. Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. *Blood* 102:336-343.

21. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U.V., Chen, X.Z., Wang, Y., Brubaker, R.F., and Hediger, M.A. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* 399:70-75.

22. Welch, R.W., Bergsten, P., Butler, J.D., and Levine, M. 1993. Ascorbic acid accumulation and transport in human fibroblasts. *Biochem J* 294 (Pt 2):505-510.

23. Corpe, C.P., Eck, P., Wang, J., Al-Hasani, H., and Levine, M. 2013. Intestinal dehydroascorbic acid (DHA) transport mediated by the facilitative sugar transporters, GLUT2 and GLUT8. *J Biol Chem* 288:9092-9101.

24. Montel-Hagen, A., Kinet, S., Manel, N., Mongellaz, C., Prohaska, R., Battini, J.L., Delaunay, J., Sitbon, M., and Taylor, N. 2008. Erythrocyte Glut1 triggers

dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. *Cell* 132:1039-1048.

25. Rumsey, S.C., Daruwala, R., Al-Hasani, H., Zarnowski, M.J., Simpson, I.A., and Levine, M. 2000. Dehydroascorbic acid transport by GLUT4 in Xenopus oocytes and isolated rat adipocytes. *J Biol Chem* 275:28246-28253.

26. Rumsey, S.C., Kwon, O., Xu, G.W., Burant, C.F., Simpson, I., and Levine, M. 1997. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem* 272:18982-18989.

27. Chatterjee, M., Saluja, R., Kumar, V., Jyoti, A., Kumar Jain, G., Kumar Barthwal, M., and Dikshit, M. 2008. Ascorbate sustains neutrophil NOS expression, catalysis, and oxidative burst. *Free Radic Biol Med* 45:1084-1093.

28. Nualart, F.J., Rivas, C.I., Montecinos, V.P., Godoy, A.S., Guaiquil, V.H., Golde, D.W., and Vera, J.C. 2003. Recycling of vitamin C by a bystander effect. *J Biol Chem* 278:10128-10133.

29. Levine, M., Dhariwal, K.R., Washko, P.W., Welch, R.W., and Wang, Y. 1993. Cellular functions of ascorbic acid: a means to determine vitamin C requirements. *Asia Pac J Clin Nutr* 2 Suppl 1:5-13.

30. Harrison, F.E., and May, J.M. 2009. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. *Free Radic Biol Med* 46:719-730.

31. Wu, X.H., and Freeze, H.H. 2002. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80:553-557.

32. Rumsey, S.C., Kwon, O., Xu, G.W., Burant, C.F., Simpson, I., and Levine, M. 1997. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *Journal of Biological Chemistry* 272:18982-18989.

33. Amir Shaghaghi, M., Bernstein, Charles N., Serrano Leo´n, Alejandra., El-Gabalawy, Hani., Eck, Peter. 2014. Polymorphisms in the sodium-dependent ascorbate transporter gene SLC23A1 are associated with susceptibility to Crohn disease. *Am J Clin Nutr* 99:378–383.

34. Silverberg, M.S., Satsangi, J., Ahmad, T., Arnott, I.D., Bernstein, C.N., Brant, S.R., Caprilli, R., Colombel, J.F., Gasche, C., Geboes, K., et al. 2005. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 19 Suppl A:5-36.

35. Ediger, J.P., Walker, J.R., Graff, L., Lix, L., Clara, I., Rawsthorne, P., Rogala, L., Miller, N., McPhail, C., Deering, K., et al. 2007. Predictors of medication adherence in inflammatory bowel disease. *Am J Gastroenterol* 102:1417-1426.

36. Washko, P.W., Hartzell, W.O., and Levine, M. 1989. Ascorbic acid analysis using high-performance liquid chromatography with coulometric electrochemical detection. *Anal Biochem* 181:276-282.

37. Soreq, H., and Seidman, S. 1992. Xenopus oocyte microinjection: from gene to protein. *Methods Enzymol* 207:225-265.

38. National Institute of Environmental Health Sciences. LD TAG SNP Selection (TagSNP).

39. Barrett, J.C., Fry, B., Maller, J., and Daly, M.J. 2005. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263-265.

40. Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., et al. 2002. The structure of haplotype blocks in the human genome. *Science* 296:2225-2229.

41. Buffinton, G.D., and Doe, W.F. 1995. Altered ascorbic acid status in the mucosa from inflammatory bowel disease patients. *Free Radic Res* 22:131-143.

42. Wendland, B.E., Aghdassi, E., Tam, C., Carrrier, J., Steinhart, A.H., Wolman, S.L., Baron, D., and Allard, J.P. 2001. Lipid peroxidation and plasma antioxidant micronutrients in Crohn disease. *Am J Clin Nutr* 74:259-264.

43. Fernandez-Banares, F., Abad-Lacruz, A., Xiol, X., Gine, J.J., Dolz, C., Cabre, E., Esteve, M., Gonzalez-Huix, F., and Gassull, M.A. 1989. Vitamin status in patients with inflammatory bowel disease. *Am J Gastroenterol* 84:744-748.

44. Hoffenberg, E.J., Deutsch, J., Smith, S., and Sokol, R.J. 1997. Circulating antioxidant concentrations in children with inflammatory bowel disease. *Am J Clin Nutr* 65:1482-1488.

45. Chapple, I.L., Matthews, J.B., Wright, H.J., Scott, A.E., Griffiths, H.R., and Grant, M.M. 2013. Ascorbate and alpha-tocopherol differentially modulate reactive oxygen species generation by neutrophils in response to FcgammaR and TLR agonists. *Innate Immun* 19:152-159.

46. Ki, M.R., Lee, H.R., Park, J.K., Hong, I.H., Han, S.Y., You, S.Y., Lee, E.M., Kim, A.Y., Lee, S.S., and Jeong, K.S. 2011. Ascorbate promotes carbon tetrachloride-induced hepatic injury in senescence marker protein 30-deficient mice by enhancing inflammation. *J Nutr Biochem* 22:535-542.

47. Ryan, J.D., Silverberg, M.S., Xu, W., Graff, L.A., Targownik, L.E., Walker, J.R., Carr, R., Clara, I., Miller, N., Rogala, L., et al. 2013. Predicting complicated Crohn's disease and surgery: phenotypes, genetics, serology and psychological characteristics of a population-based cohort. *Aliment Pharmacol Ther* 38:274-283.

CHAPTER 8

OVERALL CONCLUSION

8.1 SUMMARY AND IMPLICATIONS

The results of the present work have implications for developing genotype specific dietary interventions for patients with IBD. The entirety of the present research is the first to investigate the association of vitamin C transporters with IBD. More specifically, several variants in two different vitamin C transporter genes (*SLC23A1* and *SLC2A14*) were determined which are associated with increased risk of Crohn's disease and/or ulcerative colitis. In addition, the present research advances our understanding of the gene structure, splice variants and function of the encoded transcripts. These finding confirm our hypothesis that a disturbance in the intracellular vitamin C concentrations results in antioxidant imbalance and could contribute to the development of IBD.

In the present research, detection of genetic alterations in two different vitamin C transporter genes (*SLC23A1*and *SLC2A14)* in individuals with CD and/or UC may suggest that the observation of low ASC levels in inflamed mucosa of individuals with IBD (1) may be as much cause of inflammation as effect. It has previously been shown that the antioxidants system in intestine of individuals with IBD is impaired(1-3), and the elevated oxidative stress affects both the lamina propria cells and the function of the epithelial cells (2) and results in reduced barrier function of the gastrointestinal epithelium (4, 5). Low mucosa tissue levels (35-73%) and also low plasma concentrations of vitamin C have been reported in individuals with IBD (3, 6-8). Moreover, supplementation of antioxidant vitamins including vitamin C resulted in decrease in

indices of oxidative stress (9). In individuals with IBD, several mechanisms such as sustained production of ROS and subsequent increased in requirements, reduced oral intake, and malabsorption lead to the decreased in plasma and cellular tissue vitamin C levels. Current research showed that those individuals expressing particular variant in the two vitamin C transporter genes (*SLC23A1, SLC2A14*) may experiencing a decreased in vitamin C cellular uptake into the enterocyte or immune cells through the diminished transporter protein which results in reduced intracellular antioxidant capacity and impaired the barrier function of intestinal epithelium.

Here, for the first time, we identified that rs10063949-G allele in *SLC23A1* gene is associated with an increased CD risk. Two thirds of persons with CD carried the G allele which corresponded to 2.5 times increase in risk of CD. The genomic *SLC23A1* locus had been defined (10). However, in the current research project we update and re-define the *SLC23A1* genomic locus and its encoded transcripts. These findings expand the current knowledge on the SLC23A1 transporter gene and its disease association and provide opportunity for more biological and clinical studies.

The present study provides novel information about GLUT 14 transporter. The *SLC2A14* gene, encodes for GLUT14, was previously described as an orphan member of the facilitated membrane glucose transporter family which is exclusively expressed in primate testis (11). Here we showed that GLUT14 is a plasma membrane transporter and is expressed in extra-testicular tissues, notably in intestinal segments. We also show that GLUT14 mediates the uptake of DHA. Furthermore, for the first time, we identified three SNPs (rs12815313, rs10846086, rs2889504)

in the *SLC2A14* gene which are associated with the risk for CD and UC in a representative Caucasian cohort. The three identified SNPs are located in the intronic region of *SLC2A14* gene, thus they don't modify the encoded protein directly and thus may be a surrogate marker in LD with other variants in the same gene. The presented evidence indicate that functional SNPs in the *SLC2A14* gene contribute to an vitamin C imbalance in mucosal cells which contributes to elevated risks of IBD.

Taken together, we speculate that a deregulation of *SLC23A1* or *SLC2A14* in individuals carrying the risk genotypes lead to decreased ASC or DHA, respectively, uptake into the enterocyte or immune cells through the diminished transporter protein. Decreased intra-cellular ascorbic acid results in reduced antioxidant capacity which predisposes intestinal epithelial cells to be more vulnerable to oxidant injury in individuals with CD.

8.2 LIMITATIONS

A PhD project with the limited time and funding would not be able to provide answers to all the research questions, which was the case with the present body of research. The following areas would be considered as the main limitations of the present work:

1) Due to the nature of this research, secondary analysis of an existing cohort, dietary vitamin C intake or plasma ascorbate levels were not assessed.
2) Intestinal biopsy samples were not available to determined cell/tissue specific vitamin C levels.

3) The sample size of this study was modest, compared to other GWAS study, however the advantages were that it was a well phenotyped population based cohort.

8.3 FUTURE DIRECTIONS

Based on the novelty outcomes of this work, while considering its limitations, it is worth a further sub-grouping of the study population(s), based on SNPs described in this research, to detect how these mutations might impact vulnerability to IBD. The future study(s) should consider assessment of dietary vitamin C intake as well as serial testing of circulating/cellular ASC levels, considering that gut inflammation may change over time and hence impact on intestinal vitamin C absorption.

8.4 FINAL REMARKS

Although, the current research cannot help us to directly explain the underlying mechanism of association between vitamin C transporters and risk of IBD, it suggests that oxidative tissue damage in IBD may occur in a part as a result of disturbance in intercellular vitamin C recycling. The fact that we have observed strong associations between two different vitamin C transporters strongly suggests that a large fraction of observed abridged vitamin C in inflamed mucosa of individuals with IBD may not be secondary to oxidative injury. Thus it is possible that variations in vitamin C transporters in patients with IBD disturb intracellular redox balance and make their mucosa susceptible to initiate an inflammatory cascade and further cause additional injurious factors that can increase tissue disruption.

Upon further validation through biological and clinical studies, disturbed vitamin C balance could be restored through either ASC or DHA supplementation and thus allow personalized dietary recommendation on the basis of genotyping. Thus far, immune suppressor drugs have been the key, non invasive, therapy employed for treatment of IBD. However, due to the side effects associated with prolong usage as well as their expensive health cost (12), a safer and cheaper dietary intervention therapy would be certainly appreciated. As such, although mutations of vitamin C transporter genes may impact a relatively small group of the individuals with IBD, future personalized vitamin C dietary recommendation will be precious to ameliorate IBD severity for those individuals carrying risk genotypes.

8.5 REFERNCES

1. Head, K.A., and Jurenka, J.S. 2003. Inflammatory bowel disease Part 1: ulcerative colitis--pathophysiology and conventional and alternative treatment options. *Altern Med Rev* 8:247-283.

2. Kruidenier, L., and Verspaget, H.W. 1998. Antioxidants and mucosa protectives: realistic therapeutic options in inflammatory bowel disease? *Mediators Inflamm* 7:157-162.

3. Wendland, B.E., Aghdassi, E., Tam, C., Carrrier, J., Steinhart, A.H., Wolman, S.L., Baron, D., and Allard, J.P. 2001. Lipid peroxidation and plasma antioxidant micronutrients in Crohn disease. *Am J Clin Nutr* 74:259-264.

4. John, L.J., Fromm, M., and Schulzke, J.D. 2011. Epithelial Barriers in Intestinal Inflammation. *Antioxidants & Redox Signaling* 15:1255-1270.

5. Christophi, G.P., Rong, R., Holtzapple, P.G., Massa, P.T., and Landas, S.K. 2012. Immune markers and differential signaling networks in ulcerative colitis and Crohn's disease. *Inflammatory Bowel Diseases* 18:2342-2356.

6. Buffinton, G.D., and Doe, W.F. 1995. Altered ascorbic acid status in the mucosa from inflammatory bowel disease patients. *Free Radic Res* 22:131-143.

7. Fernandez-Banares, F., Abad-Lacruz, A., Xiol, X., Gine, J.J., Dolz, C., Cabre, E., Esteve, M., Gonzalez-Huix, F., and Gassull, M.A. 1989. Vitamin status in patients with inflammatory bowel disease. *Am J Gastroenterol* 84:744-748.

8. Hoffenberg, E.J., Deutsch, J., Smith, S., and Sokol, R.J. 1997. Circulating antioxidant concentrations in children with inflammatory bowel disease. *Am J Clin Nutr* 65:1482-1488.

9. Aghdassi, E., Wendland, B.E., Steinhart, A.H., Wolman, S.L., Jeejeebhoy, K., and Allard, J.P. 2003. Antioxidant vitamin supplementation in Crohn's disease decreases oxidative stress. a randomized controlled trial. *Am J Gastroenterol* 98:348-353.

10. Eck, P., Erichsen, H.C., Taylor, J.G., Yeager, M., Hughes, A.L., Levine, M., and Chanock, S. 2004. Comparison of the genomic structure and variation in the two human sodium-dependent vitamin C transporters, SLC23A1 and SLC23A2. *Hum Genet* 115:285-294.

11. Wu, X., and Freeze, H.H. 2002. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 80:553-557.

12. Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., et al. 2012. Increasing

incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 142:46-54 e42; quiz e30.

APPENDICES

APPENDIX I

COPYRIGHT LICENSE FOR PREVIOUSLY PUBLISHED MATERIAL

RightsLink®

Home | Create Account | Help | Live Chat

Title: Polymorphisms in the sodium-dependent ascorbate transporter gene SLC23A1 are associated with susceptibility to Crohn disease

Author: Mandana Amir Shaghaghi, Charles N Bernstein, Alejandra Serrano León, Hani El-Gabalawy, Peter Eck

Publication: The American Journal of Clinical Nutrition

Publisher: American Society for Nutrition

Date: Feb 1, 2014

Copyright © 2014, The American Journal of Clinical Nutrition

LOGIN

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already **a RightsLink user** or want to learn more?

Retained Author Rights

An author of an ASN article retains the right to include his/her article in his/her thesis or dissertation. Authors may post a link on a personal website that directs readers to the article on The American Journal of Clinical Nutrition website (www.ajcn.org); full text of the final, published article cannot be posted on personal or institutional websites or repositories that are accessible to the public.

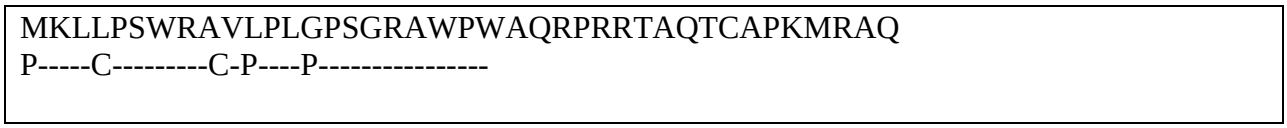
BACK | CLOSE WINDOW

Appendix II

ADDITIONAL INFORMATION, TABLES, AND RESULTS CORRESPONDING TO STUDIES DESCRIBED IN CHAPTERS IV, VI, AND VII

CHAPTER IV SUPPLEMENT

Supplement Figure 1: The novel SLC23A1 Exon 1A splices into the previously described Exon 1B to encode a protein which has 36 additional N-terminal amino acids. SLC23A1 Exon 1A nucleotides are depicted in blue, nucleotides of the 5' terminus of Exon 1B in black, and the translated protein in brown. The novel and the previously known start codons are marked in bold.



Supplement Figure 2: Three novel protein-protein binding sites are predicted in the novel N-terminus of SLC23A1 protein isoform 1A (Predict Protein), indicated by the letter P in the second row. Two additional Protein kinase C phosphorylation sites are predicted, indicated by C in the second row (ProSite).

SLC23A1 splice variant 1A transcript (2473 nucleotides):

AAAACTGGTCCAGCAACTTTGGAAATTGGGAGCAGTAGCCAAGAGGGAGAGGAGA
GGACAGGCGGACAGAGGAACAGTGTGGACACCGGCAGGCGGGCGGACTCAGTGCT
GTGGCATGAAGCTGCTGCCCAGCTGGAGAGCGGTGCTGCCCCTTGGCCCTTCAGGCA
GGGCCTGGCCATGGGCACAGCGACCCAGAAGAACTGCTCAAACCTGTGCCCCAAAG

ATGAGGGCCCAGGAGGACCTCGAGGGCCGGACACAGCATGAAACCACCAGGGACC
CCTCGACCCCGCTACCCACAGAGCCTAAGTTTGACATGTTGTACAAGATCGAGGACG
TGCCACCTTGGTACCTGTGCATCCTGCTGGGCTTCCAGCACTACCTGACATGCTTCA
GTGGTACCATCGCCGTGCCCTTCCTGCTGGCTGAGGCGCTGTGTGTGGGCCACGACC
AGCACATGGTTAGTCAGCTCATCGGCACCATCTTCACGTGCGTGGGCATCACCACTC
TCATCCAGACCACCGTGGGCATCCGGCTGCCGCTGTTCCAGGCCAGTGCCTTTGCAT
TTCTGGTTCCAGCCAAAGCCATACTGGCTCTGGAGAGATGGAAATGCCCCCCGGAA
GAGGAGATCTACGGTAACTGGAGTCTGCCCCTGAACACCTCTCATATTTGGCACCCA
CGGATACGGGAGGTCCAGGGTGCAATCATGGTGTCCAGCGTGGTGGAGGTGGTGAT
TGGCCTGCTGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTCTCACAGTCAC
CCCCACTGTCTCCCTCATTGGCCTTTCTGTCTTCCAAGCTGCTGGCGACCGAGCTGGC
TCCCACTGGGGCATCTCAGCTTGCTCCATTCTCCTGATCATCCTCTTCTCCCAGTACC
TGCGCAACCTCACCTTCCTGCTGCCTGTCTACCGCTGGGGCAAGGGCCTCACTCTCC
TCCGCATCCAGATCTTCAAAATGTTTCCTATCATGCTGGCCATCATGACCGTGTGGCT
GCTCTGCTATGTCCTGACCTTGACAGACGTGCTGCCCACAGACCCAAAAGCCTATGG
CTTCCAGGCACGAACCGATGCCCGTGGTGACATCATGGCTATTGCACCCTGGATCCG
CATCCCCTACCCCTGTCAGTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGGG
AATGTTCAGCGCCACTCTGGCAGGCATCATTGAGTCCATCGGAGATTACTACGCCTG
TGCCCGCCTGGCTGGTGCACCACCCCCTCCAGTACATGCTATCAACAGGGGCATCTT
CACCGAAGGCATTTGCTGCATCATCGCGGGGCTATTGGGCACGGGCAACGGGTCCA
CCTCGTCCAGTCCCAACATTGGCGTCCTGGGAATTACCAAGGTGGGCAGCCGGCGC
GTGGTGCAGTATGGTGCGGCTATCATGCTGGTCCTGGGCACCATCGGCAAGTTCACG
GCCCTCTTCGCCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTTG
GCATGATTACAGCTGTGGGGCTGTCCAACCTGCAATTTGTGGACATGAACTCCTCTC
GCAACCTCTTCGTGCTGGGATTTTCCATGTTCTTCGGGCTCACGCTGCCCAATTACCT
GGAGTCCAACCCTGGCGCCATCAATACAGGCATTCTTGAAGTGGATCAGATTCTGAT
TGTGCTGCTGACCACGGAGATGTTTGTGGGCGGGTGCCTTGCTTTCATACTTGACAA
CACAGTGCCAGGGAGCCCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGGGGCTC
ATGCCAACAGTGACATGTCTTCCAGCCTCAAGAGCTACGATTTCCCCATTGGGATGG
GCATAGTAAAAAGAATTACCTTTCTGAAATACATTCCTATCTGCCCAGTCTTCAAAG
GATTTTCTTCAAGTTCAAAAGATCAGATTGCAATTCCAGAAGACACTCCAGAAAATA
CAGAAACTGCATCTGTGTGCACCAAGGTCTGAAAAATGACTTCCAGGAAAGGAAGC
ATGGTATATAACAGGAAAAGAAAACTACATGGGGAACCAGAAGACCTAAGCCTGA
AATCCCAGCCCTGCCCCTAACTAACTTCTGTGTAAACTCAGATAAGTCACCTTTCTCT
GGGATTCAAATTTTTGCATCAGTTAAAAAAAAAGGGGTGGGGGGGAATGGGCCAAA
GTCTGAGTCTTAGAGACTTGTACCAATGTTATGCTATGTCTCTAAATCTTTACTCTCC
TAAGTAGACTTGTCAGCATCTAGGAAGAACAGCTAGAAATTTTCCTCTGTGATATTT
TAGACTGCAAGTTGAAAAAAATAAAAAGAAATGAGGGCAGGTTCCAGGGCCTGAA
ATGTAGGTATGCTGCAAGGCTTTTACATTGAATTTGACCCTACATCACTTCAAGACT
AATGCATAATATTAAACATCATGTTGAAGAAAT

SLC23A1 splice variant 1A ORF (1905 nucleotides):

ATGAAGCTGCTGCCCAGCTGRAGAGCGGTGCTGCCCCTTGGCCCTTCAGGCAGGGCC
TGGCCATGGGCACAGCGACCCAGAAGAACTGCTCAAACCTGTGCCCCAAAGATGAG

GGCCCAGGAGGACCTCGAGGGCCGGACACAGCATGAAACCACCAGGGACCCCTCG
ACCCCGCTACCCACAGAGCCTAAGTTTGACATGTTGTACAAGATCGAGGACGTGCC
ACCTTGGTACCTGTGCATCCTGCTGGGCTTCCAGCACTACCTGACATGCTTCAGTGGT
ACCATCGCCGTGCCCTTCCTGCTGGCTGAGGCGCTGTGTGTGGGCCACGACCAGCAC
ATGGTTAGTCAGCTCATCGGCACCATCTTCACGTGCGTGGGCATCACCACTCTCATC
CAGACCACCGTGGGCATCCGGCTGCCGCTGTTCCAGGCCAGTGCCTTTGCATTTCTG
GTTCCAGCCAAAGCCATACTGGCTCTGGAGAGATGGAAATGCCCCCCGGAAGAGGA
GATCTACGGTAACTGGAGTCTGCCCCTGAACACCTCTCATATTTGGCACCCACGGAT
ACGGGAGGTCCAGGGTGCAATCATGGTGTCCAGCGTGGTGGAGGTGGTGATTGGCC
TGCTGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTCTCACAGTCACCCCCA
CTGTCTCCCTCATTGGCCTTTCTGTCTTCCAAGCTGCTGGCGACCGAGCTGGCTCCCA
CTGGGGCATCTCAGCTTGCTCCATTCTCCTGATCATCCTCTTCTCCCAGTACCTGCGC
AACCTCACCTTCCTGCTGCCTGTCTACCGCTGGGGCAAGGGCCTCACTCTCCTCCGC
ATCCAGATCTTCAAAATGTTTCCTATCATGCTGGCCATCATGACCGTGTGGCTGCTCT
GCTATGTCCTGACCTTGACAGACGTGCTGCCCACAGACCCAAAAGCCTATGGCTTCC
AGGCACGAACCGATGCCCGTGGTGACATCATGGCTATTGCACCCTGGATCCGCATCC
CCTACCCCTGTCAGTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGGGAATGT
TCAGCGCCACTCTGGCAGGCATCATTGAGTCCATCGGAGATTACTACGCCTGTGCCC
GCCTGGCTGGTGCACCACCCCCTCCAGTACATGCTATCAACAGGGGCATCTTCACCG
AAGGCATTTGCTGCATCATCGCGGGGCTATTGGGCACGGGCAACGGGTCCACCTCGT
CCAGTCCCAACATTGGCGTCCTGGGAATTACCAAGGTGGGCAGCCGGCGCGTGGTG
CAGTATGGTGCGGCTATCATGCTGGTCCTGGGCACCATCGGCAAGTTCACGGCCCTC
TTCGCCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTTGGCATGA
TTACAGCTGTGGGGCTGTCCAACCTGCAATTTGTGGACATGAACTCCTCTCGCAACC
TCTTCGTGCTGGGATTTTCCATGTTCTTCGGGCTCACGCTGCCCAATTACCTGGAGTC
CAACCCTGGCGCCATCAATACAGGCATTCTTGAAGTGGATCAGATTCTGATTGTGCT
GCTGACCACGGAGATGTTTGTGGGCGGGTGCCTTGCTTTCATACTTGACAACACAGT
GCCAGGGAGCCCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGGGGCTCATGCCA
ACAGTGACATGTCTTCCAGCCTCAAGAGCTACGATTTCCCCATTGGGATGGGCATAG
TAAAAAGAATTACCTTTCTGAAATACATTCCTATCTGCCCAGTCTTCAAAGGATTTTC
TTCAAGTTCAAAAGATCAGATTGCAATTCCAGAAGACACTCCAGAAAATACAGAAA
CTGCATCTGTGTGCACCAAGGTCTGA

SLC23A1 splice variant 1B transcript (2355 nucleotides):

AGATTTACTTCAAACAATGCCTGATATGTGGAGTAAAGTCCTCCTCCTTGGCCTTTGT
CAAGTCATCCCCTCTTCTCCTCAGGAACTGCTCAAACCTGTGCCCCAAAGATGAGGG
CCCAGGAGGACCTCGAGGGCCGGACACAGCATGAAACCACCAGGGACCCCTCGACC
CCGCTACCCACAGAGCCTAAGTTTGACATGTTGTACAAGATCGAGGACGTGCCACCT
TGGTACCTGTGCATCCTGCTGGGCTTCCAGCACTACCTGACATGCTTCAGTGGTACC
ATCGCCGTGCCCTTCCTGCTGGCTGAGGCGCTGTGTGTGGGCCACGACCAGCACATG
GTTAGTCAGCTCATCGGCACCATCTTCACGTGCGTGGGCATCACCACTCTCATCCAG
ACCACCGTGGGCATCCGGCTGCCGCTGTTCCAGGCCAGTGCCTTTGCATTTCTGGTT
CCAGCCAAAGCCATACTGGCTCTGGAGAGATGGAAATGCCCCCCGGAAGAGGAGAT

CTACGGTAACTGGAGTCTGCCCCTGAACACCTCTCATATTTGGCACCCACGGATACG
GGAGGTCCAGGGTGCAATCATGGTGTCCAGCGTGGTGGAGGTGGTGATTGGCCTGC
TGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTCTCACAGTCACCCCCACTG
TCTCCCTCATTGGCCTTTCTGTCTTCCAAGCTGCTGGCGACCGAGCTGGCTCCCACTG
GGGCATCTCAGCTTGCTCCATTCTCCTGATCATCCTCTTCTCCCAGTACCTGCGCAAC
CTCACCTTCCTGCTGCCTGTCTACCGCTGGGGCAAGGGCCTCACTCTCCTCCGCATCC
AGATCTTCAAAATGTTTCCTATCATGCTGGCCATCATGACCGTGTGGCTGCTCTGCTA
TGTCCTGACCTTGACAGACGTGCTGCCCACAGACCCAAAAGCCTATGGCTTCCAGGC
ACGAACCGATGCCCGTGGTGACATCATGGCTATTGCACCCTGGATCCGCATCCCCTA
CCCCTGTCAGTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGGGAATGTTCAG
CGCCACTCTGGCAGGCATCATTGAGTCCATCGGAGATTACTACGCCTGTGCCCGCCT
GGCTGGTGCACCACCCCCTCCAGTACATGCTATCAACAGGGGCATCTTCACCGAAGG
CATTTGCTGCATCATCGCGGGGCTATTGGGCACGGGCAACGGGTCCACCTCGTCCAG
TCCCAACATTGGCGTCCTGGGAATTACCAAGGTGGGCAGCCGGCGCGTGGTGCAGT
ATGGTGCGGCTATCATGCTGGTCCTGGGCACCATCGGCAAGTTCACGGCCCTCTTCG
CCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTTGGCATGATTAC
AGCTGTGGGGCTGTCCAACCTGCAATTTGTGGACATGAACTCCTCTCGCAACCTCTT
CGTGCTGGGATTTTCCATGTTCTTCGGGCTCACGCTGCCCAATTACCTGGAGTCCAAC
CCTGGCGCCATCAATACAGGCATTCTTGAAGTGGATCAGATTCTGATTGTGCTGCTG
ACCACGGAGATGTTTGTGGGCGGGTGCCTTGCTTTCATACTTGACAACACAGTGCCA
GGGAGCCCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGGGGCTCATGCCAACAG
TGACATGTCTTCCAGCCTCAAGAGCTACGATTTCCCCATTGGGATGGGCATAGTAAA
AAGAATTACCTTTCTGAAATACATTCCTATCTGCCCAGTCTTCAAAGGATTTTCTTCA
AGTTCAAAAGATCAGATTGCAATTCCAGAAGACACTCCAGAAAATACAGAAACTGC
ATCTGTGTGCACCAAGGTCTGAAAAATGACTTCCAGGAAAGGAAGCATGGTATATA
ACAGGAAAAGAAAACTACATGGGGAACCAGAAGACCTAAGCCTGAAATCCCAGCC
CTGCCCCTAACTAACTTCTGTGTAAACTCAGATAAGTCACCTTTCTCTGGGATTCAAA
TTTTTGCATCAGTTAAAAAAAAAGGGGTGGGGGGGAATGGGCCAAAGTCTGAGTCT
TAGAGACTTGTACCAATGTTATGCTATGTCTCTAAATCTTTACTCTCCTAAGTAGACT
TGTCAGCATCTAGGAAGAACAGCTAGAAATTTTCCTCTGTGATATTTTAGACTGCAA
GTTGAAAAAAATAAAAAGAAATGAGGGCAGGTTCCAGGGCCTGAAATGTAGGTATG
CTGCAAGGCTTTTACATTGAATTTGACCCTACATCACTTCAAGACTAATGCATAATA
TTAAACATCATGTTGAAGA

SLC23A1 splice variant 1B ORF:

ATGAGGGCCCAGGAGGACCTCGAGGGCCGGACACAGCATGAAACCACCAGGGACC
CCTCGACCCCGCTACCCACAGAGCCTAAGTTTGACATGTTGTACAAGATCGAGGACG
TGCCACCTTGGTACCTGTGCATCCTGCTGGGCTTCCAGCACTACCTGACATGCTTCA
GTGGTACCATCGCCGTGCCCTTCCTGCTGGCTGAGGCGCTGTGTGTGGGCCACGACC
AGCACATGGTTAGTCAGCTCATCGGCACCATCTTCACGTGCGTGGGCATCACCACTC
TCATCCAGACCACCGTGGGCATCCGGCTGCCGCTGTTCCAGGCCAGTGCCTTTGCAT
TTCTGGTTCCAGCCAAAGCCATACTGGCTCTGGAGAGATGGAAATGCCCCCCGGAA
GAGGAGATCTACGGTAACTGGAGTCTGCCCCTGAACACCTCTCATATTTGGCACCCA

CGGATACGGGAGGTCCAGGGTGCAATCATGGTGTCCAGCGTGGTGGAGGTGGTGAT
TGGCCTGCTGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTCTCACAGTCAC
CCCCACTGTCTCCCTCATTGGCCTTTCTGTCTTCCAAGCTGCTGGCGACCGAGCTGGC
TCCCACTGGGGCATCTCAGCTTGCTCCATTCTCCTGATCATCCTCTTCTCCCAGTACC
TGCGCAACCTCACCTTCCTGCTGCCTGTCTACCGCTGGGGCAAGGGCCTCACTCTCC
TCCGCATCCAGATCTTCAAAATGTTTCCTATCATGCTGGCCATCATGACCGTGTGGCT
GCTCTGCTATGTCCTGACCTTGACAGACGTGCTGCCCACAGACCCAAAAGCCTATGG
CTTCCAGGCACGAACCGATGCCCGTGGTGACATCATGGCTATTGCACCCTGGATCCG
CATCCCCTACCCCTGTCAGTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGGG
AATGTTCAGCGCCACTCTGGCAGGCATCATTGAGTCCATCGGAGATTACTACGCCTG
TGCCCGCCTGGCTGGTGCACCACCCCCTCCAGTACATGCTATCAACAGGGGCATCTT
CACCGAAGGCATTTGCTGCATCATCGCGGGGCTATTGGGCACGGGCAACGGGTCCA
CCTCGTCCAGTCCCAACATTGGCGTCCTGGGAATTACCAAGGTGGGCAGCCGGCGC
GTGGTGCAGTATGGTGCGGCTATCATGCTGGTCCTGGGCACCATCGGCAAGTTCACG
GCCCTCTTCGCCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTTG
GCATGATTACAGCTGTGGGGCTGTCCAACCTGCAATTTGTGGACATGAACTCCTCTC
GCAACCTCTTCGTGCTGGGATTTTCCATGTTCTTCGGGCTCACGCTGCCCAATTACCT
GGAGTCCAACCCTGGCGCCATCAATACAGGCATTCTTGAAGTGGATCAGATTCTGAT
TGTGCTGCTGACCACGGAGATGTTTGTGGGCGGGTGCCTTGCTTTCATACTTGACAA
CACAGTGCCAGGGAGCCCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGGGGCTC
ATGCCAACAGTGACATGTCTTCCAGCCTCAAGAGCTACGATTTCCCCATTGGGATGG
GCATAGTAAAAAGAATTACCTTTCTGAAATACATTCCTATCTGCCCAGTCTTCAAAG
GATTTTCTTCAAGTTCAAAAGATCAGATTGCAATTCCAGAAGACACTCCAGAAAATA
CAGAAACTGCATCTGTGTGCACCAAGGTCTGA

SLC23A1 protein isoform B (shorter). Protein sequence of the SLC23A1 short isoform B

representing the previously described protein NP689898

MRAQEDLEGRTQHETTRDPSTPLPTEPKFDMLYKIEDVPPWYLCILLGFQHYLTCFSGTI
AVPFLLAEALCVGHDQHMVSQLIGTIFTCVGITTLIQTTVGIRLPLFQASAFAFLVPAKAIL
ALERWKCPPEEEIYGNWSLPLNTSHIWHPRIREVQGAIMVSSVVEVVIGLLGLPGALLNY
IGPLTVTPTVSLIGLSVFQAAGDRAGSHWGISACSILLIILFSQYLRNLTFLLPVYRWGKGL
TLLRIQIFKMFPIMLAIMTVWLLCYVLTLTDVLPTDPKAYGFQARTDARGDIMAIAPWIR
IPYPCQWGLPTVTAAAVLGMFSATLAGIIESIGDYYACARLAGAPPPPVHAINRGIFTEGI
CCIIAGLLGTGNGSTSSSPNIGVLGITKVGSRRVVQYGAAIMLVLGTIGKFTALFASLPDPI
LGGMFCTLFGMITAVGLSNLQFVDMNSSRNLFVLGFSMFFGLTLPNYLESNPGAINTGIL
EVDQILIVLLTTEMFVGGCLAFILDNTVPGSPEERGLIQWKAGAHANSDMSSSLKSYDFP
IGMGIVKRITFLKYIPICPVFKGFSSSSKDQIAIPEDTPENTETASVCTKV.

SLC23A1 protein isoform A (longer)

MKLLPSWRAVLPLGPSGRAWPWAQRPRRTAQTCAPKMRAQEDLEGRTQHETTRDPST
PLPTEPKFDMLYKIEDVPPWYLCILLGFQHYLTCFSGTIAVPFLLAEALCVGHDQHMVSQ
LIGTIFTCVGITTLIQTTVGIRLPLFQASAFAFLVPAKAILALERWKCPPEEEIYGNWSLPL
NTSHIWHPRIREVQGAIMVSSVVEVVIGLLGLPGALLNYIGPLTVTPTVSLIGLSVFQAAG
DRAGSHWGISACSILLIILFSQYLRNLTFLLPVYRWGKGLTLLRIQIFKMFPIMLAIMTVW
LLCYVLTLTDVLPTDPKAYGFQARTDARGDIMAIAPWIRIPYPCQWGLPTVTAAAVLGM
FSATLAGIIESIGDYYACARLAGAPPPPVHAINRGIFTEGICCIIAGLLGTGNGSTSSSPNIG
VLGITKVGSRRVVQYGAAIMLVLGTIGKFTALFASLPDPILGGMFCTLFGMITAVGLSNL
QFVDMNSSRNLFVLGFSMFFGLTLPNYLESNPGAINTGILEVDQILIVLLTTEMFVGGCL
AFILDNTVPGSPEERGLIQWKAGAHANSDMSSSLKSYDFPIGMGIVKRITFLKYIPICPVF
KGFSSSSKDQIAIPEDTPENTETASVCTKV.

Table 1: comparison of Protein Parameters
(ProtParm [http://web.expasy.org/cgi-bin/protparam/protparam])

	SLC23A1 short isoform B	SLC23A1 long isoform A
Number of amino acids	598	634
Molecular weight	64815.1	68882
Theoretical pI	6.16	8.09
Total number of negatively charged residues Asp + Glu)	41	41
Total number of positively charged residues (Arg + Lys)	37	44
Instability index	38.19, stable	39.2, stable
Aliphatic index	109.26	106.77
grand average of hydropathicity (GRAVY)	0.497	0.436

Table 2. List of oligonucleotides (primers) used for the subcloning and fusion of and *SLC23A1* Exon 1A and turbo GFP

Name	Sequence
SLC23A1 Exon1A forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGCTGC TGCCCAGCTGGAGAGC
SLC23A1 Exon1A reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGACCTTGG TGCACACAGATG
turbo GFP *forward*	GCCACCATGGAGAGCGACGAGAGC
turbo GFP *reverse*	TCAGACCTTGGTGCACACAGATG
SLC23A1- turbo GFP overlap 1	GCAGATGCCGGTGAAGAAATGAAGCTGCTGCCCAGCTGG
SLC23A1- turbo GFP overlap 2	TCAGACCTTGGTGCACACAGATG
SLC23A1 Exon1A Gateway tagged	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCACCATGGAGAGCGACGAGAGC
turbo GFP *Gateway tagged*	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGACCTTGG TGCACACAGATG

CHAPTER VI SUPPLEMENT

SLC2A14 orthologues are only found in selected primates, demonstrated by the fact that a standard blastn search (http://blast.ncbi.nlm.nih.gov.proxy1.lib.umanitoba.ca/Blast.cgi, performed on 06-04-2015) against all deposited nucleotides using the *SLC2A14* a specific sequence (exon 1, see sequence below) revealed only hits for human, the common chimpanzee (Pan troglodytes), and the northern white-cheeked gibbon (Nomascus leucogenys) (Web Supplement Figure 1).

Sequence blasted:

ACCTCCCAATAGCCCGGCGGCCGCATGAGGCTGGGACTGGCGCACGGAGGCGGTGG
GGCGGCGGGGGGGGGGGAAGGGGGAGGGGAAGGGGGGTGCTGAAGCGGGGGGGGC
GAGAAACTGGAGACCCTGCAAAAAGGCTGGGCCTGGGCTGCTCTGGTTACCCCGCG
AGTCCTCTTCACTGCGCAGGCGTGGGCAGCTGCAGAACCCTGTCATTCTATCTTAGT
TTGCTCATCATCACTAATATTCTTTGCTATGACCCAGGATATACCTGGAACCCAGCG
ACCTTGACCTGCCTTCCAATGCAG

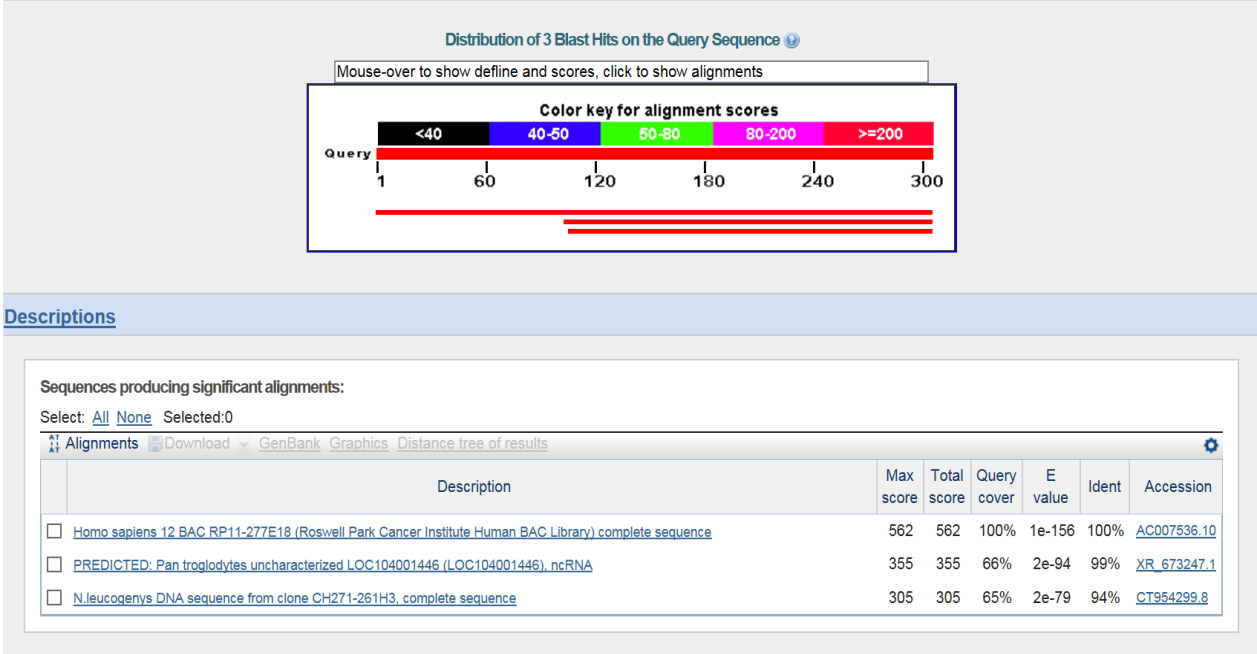


Figure 1: Screenshot of the blastn search described above.

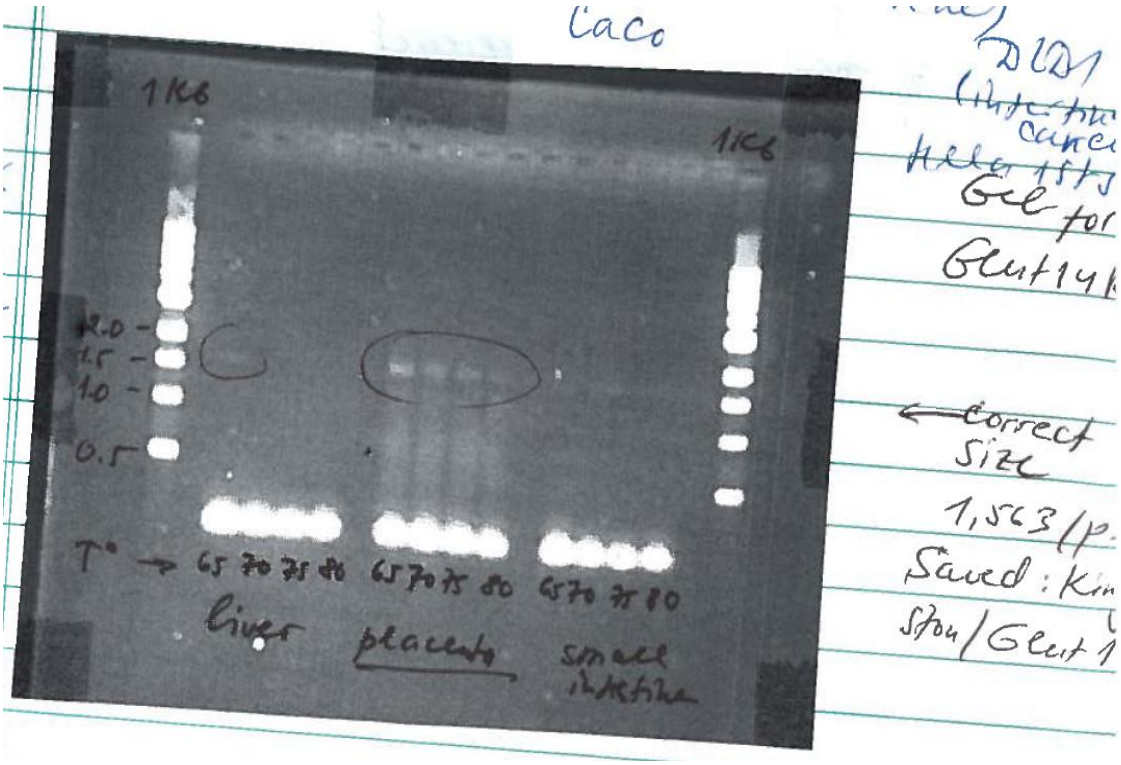


Figure 2: Expression of GLUT14 in liver, placenta and small intestine. A 1% Agarose gel stained with ethidium bromide and visualized in a Gel Doc™ imaging system (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario) shows amplicons for the GLUT14 Open Reading Frame. The identity of the amplicons was confirmed by sequencing.

Figure 3: SLC2A14 exon 5 is a transcriptional start site and alternatively spliced on its 3' end. The alignment of existing ESTs (row two onwards) toward the genomic DNA (first row) shows the origin of numerous transcripts, which are all testis specific, as well as the utilization of an alternative splice donor site, eliminating 53 bases in the 3'.

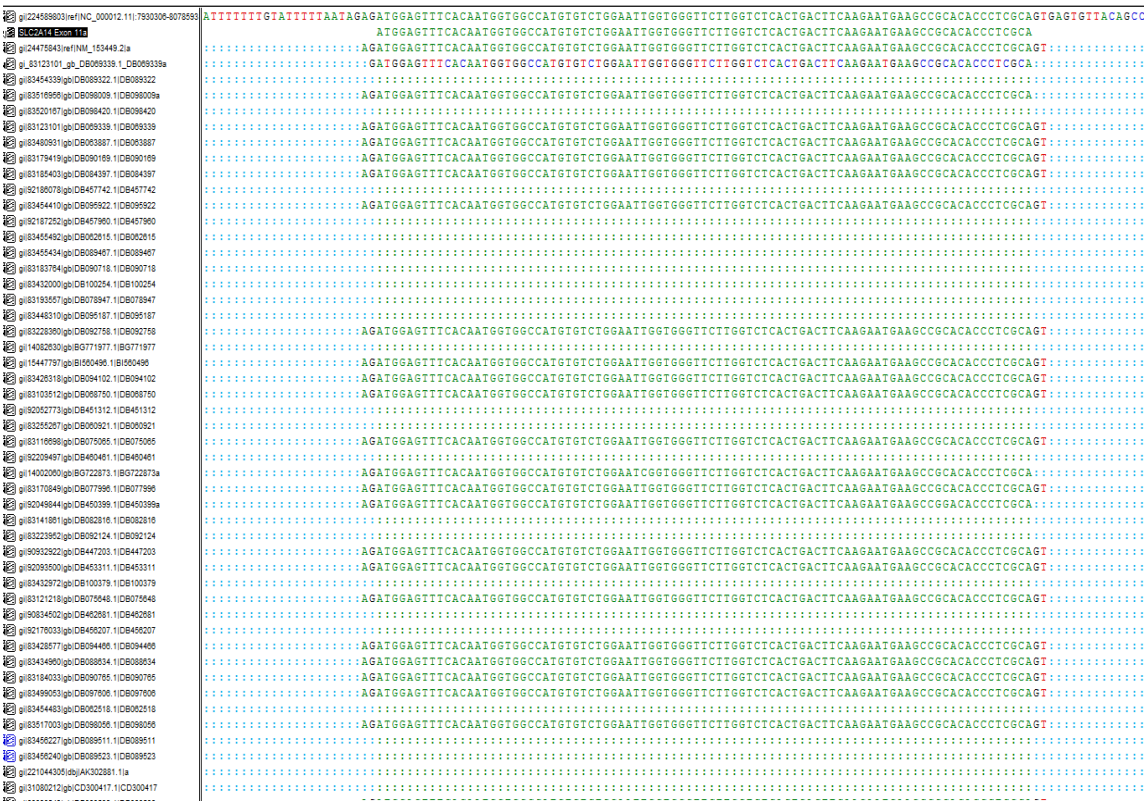


Figure 4: Example for the differential utilization of SLC2A14 exon 11 in transcripts of testicular origin.

Figure 5: Example for the differential utilization of SLC2A14 exon 13.

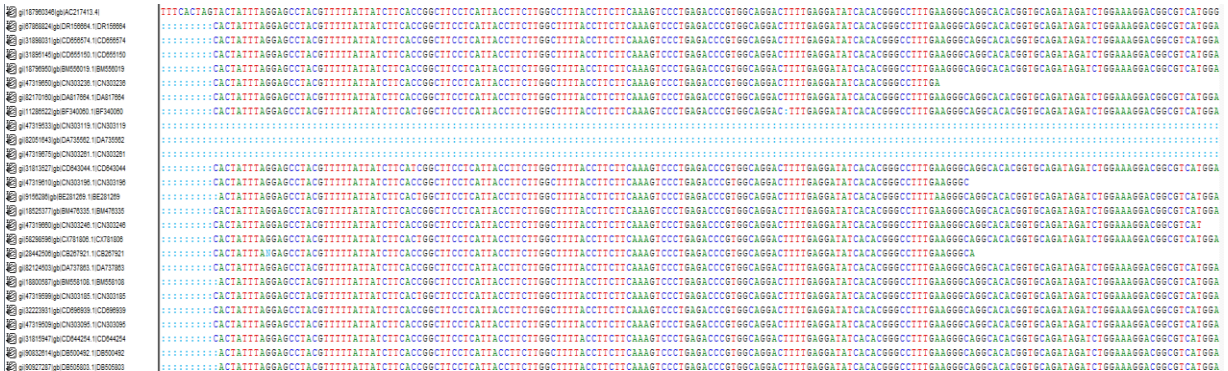


Figure 6: Example for the differential utilization in the 5’ of SLC2A14 exon 20.

CHAPTER VII SUPPLEMENT

TABLE 1: Summary of 8 tagging SNPs in *GLUT 14* gene involved in this study

SNP	SNP Type	Position (Chromosome 12)	Alleles Major/minor	*Catalog ID
rs11612319	Intron	8016925 bp	G/A	ID: C___2875382_10
rs7132415	Intron	7997547 bp	G/T	ID: C__29025049_10
rs10846086	Intron	8025266 bp	A/G	ID: C__12095733_10
rs2376904	Intron	8000995 bp	G/A	ID: C__26191692_10
rs7300773	Intron	8023943 bp	T/C	ID: C__29292592_20
rs2889504	Intron	8008132 bp	G/T	ID: C___2875393_10
rs12815313	Intron	8023530 bp	C/T	ID: C__31787372_10
rs10845990	Intron	7970721 bp	T/G	ID: C__30894136_10

Note: SNP: single nucleotide polymorphisms; bp: base pair
* TaqMan® SNP Genotyping Assay (life technologies)

TABLE 2: General characteristics of the study participants

Parameters	**Ulcerative Colitis (n= 149)**	**Crohn´s Disease (n=162)**	**Controls (n=142)**
Gender			
Female	87 (58.4%)	97 (59.9%)	80 (56.3%)
Male	62 (41.6%)	65 (40.1%)	62 (43.7%)
Age at Diagnosis			
A1(<17 yr)	12 (8.1%)	17 (10.5%)	-
A2 (17-40 yr)	78 (52.3%)	101 (62.3%)	-
A3 (>40 yr)	59 (39.6%)	44 (27.2%)	-
Disease Location			
Terminal ileum	-	69 (42.6%)	-
Colon	-	37 (22.8%)	-
Ileocolon	-	51 (31.5%)	-
Proximal GI tract	-	5 (3.1%)	-
Proctitis	11 (7.4%)	-	-
Left-sided colitis	68 (45.6%)	-	-
Pancolitis	70 (47.0%)	-	-
Disease Type			
Inflammatory	-	69 (42.6%)	-
Fibrostenotic	-	54 (33.3%)	-
Penetrating/Fistulizing	-	39 (24.1%)	-

APPENDIX III

PUBLICATION OF THESIS RELEVANCE

The American Journal of Clinical Nutrition

Single-nucleotide polymorphisms in *SLC22A23* are associated with ulcerative colitis in a Canadian white cohort[1–4]

Alejandra Serrano León, Mandana Amir Shaghaghi, Natalia Yurkova, Charles N Bernstein, Hani El-Gabalawy, and Peter Eck

ABSTRACT

Background: *SLC22A23* is an orphan gene in the *SLC22* family of organic membrane transporters, and its single-nucleotide polymorphism rs17309827-T was recently nominally associated with intestinal inflammation in a genome-wide association study. Other polymorphisms in the *SLC22A23* gene have been associated with diseases with an inflammatory component, and polymorphisms in related genes in the *SLC22* family have been repeatedly associated with inflammatory bowel disease (IBD).

Objective: In a candidate-gene study using a well-phenotyped, highly monitored, Manitoban white cohort, we investigated whether variations in *SLC22A23* were associated with intestinal inflammation.

Design: Selected genetic variations were genotyped by using fluorescent-based assays or a polymerase chain reaction–restriction fragment length polymorphism analysis in 160 individuals with Crohn disease, 149 individuals with ulcerative colitis, and 142 healthy control subjects to determine genetic associations.

Results: Homozygocity for single-nucleotide polymorphisms rs4959235-TT and rs950318-GG was associated with IBD, whereby 6% of patients (18 of 311 cases) carried these genotypes, but they were not seen in healthy controls.

Conclusion: Associations reported in this article add to the emerging evidence that *SLC22A23* variants could modify IBD risk. However, the biology of the gene and impact of variations on the gene's functions need to be tested to validate a causative role. *Am J Clin Nutr* 2014;100:289–94.

INTRODUCTION

Inflammatory bowel disease (IBD)[5] is characterized by chronic inflammation of the gastrointestinal tract with the principal forms Crohn disease (CD) and ulcerative colitis (UC). Mostly unknown environmental and genetic factors contribute to the immune dysregulation, which determine the development, maintenance, pharmacokinetics, and, thus, the bioactivity of unknown organic ions, which might modulate the susceptibility and severity of IBD or other related common and complex diseases. Hence, we hypothesized that an organic ion imbalance as a consequence of polymorphisms in transporter genes contributes to the cause of IBD.

The single-nucleotide polymorphism (SNP) rs17309827-T, which is located in a gene in the SLC22 transporter family *SLC22A23*, has been associated with IBD in a GWAS (3, 20), which has strengthened the hypothesis that imbalances of organic ions might modulate IBD risk. In addition to IBD, polymorphisms in the *SLC22A23* gene have been associated with other complex diseases that have an inflammatory component, such as endometriosis-related infertility (21), and the clearance of antipsychotic drugs (22). *SLC22A23* SNP rs17136561 was nominally associated with the development of asthma in individuals with impaired allergic status, although the results did not reach an overall genome-wide significance (OR: 1.64; 95% CI: 1.33, 2.02; $P = 2.3 \times 10^{-6}$) (23). *SLC22A23* was also 1 of 6 genes in which expressions could be used to predict the recurrence of triple-negative breast cancer in a cohort of Taiwanese women (24).

On the basis of the emerging evidence associating genetic variations in *SLC22A23* with IBD and other diseases having an inflammatory component, and because the gene is related to

[1] From Human Nutritional Sciences and the Richardson Centre for Functional Foods and Nutraceuticals (ASL, MAS, NY, and PE), the University of Manitoba Inflammatory Bowel Disease Clinical and Research Centre (CNB), and the Department of Internal Medicine (CNB and HE-G), University of Manitoba, Winnipeg, Canada.

[2] ASL and MAS contributed equally to the manuscript.

[3] Supported by a Canada Research Chair (PE), a Canadian Institutes of

APPENDIX IV

INVITATION LETTER FROM "ADVANCES IN NUTRITION"

Invitation to submit a manuscript to Advances in Nutrition

Katie Dunn Add to contacts 11/18/2014 Photos Actions

To: mani_shaghaghi@hotmail.com Cc: Karen King

Session Title: *Association of GLUT14 Genetic Variants with Risk of Inflammatory Bowel Disease in Caucasians*

Dear Dr. Amir Shaghaghi:

I am contacting you on behalf of Dr. Katherine Tucker, Editor, *Advances in Nutrition* (*AN*), who would like to encourage you to submit a manuscript to *AN* based on the topic of your presentation at the upcoming "Advances and Controversies in Clinical Nutrition 2014" conference. *Advances in Nutrition* is a review journal, with a 2013 Impact Factor of 4.89, published by the American Society for Nutrition. A finalist for the 2013 ALPSP "Best New Journal" award (http://asn-cdn-remembers.s3.amazonaws.com/14ebb03370560ac3af4ddf9dcd28f650.pdf), the journal publishes review articles that highlight the significance of recent research in nutrition and illustrate the central role of nutrition in the promotion of health and prevention of disease. On behalf of Dr. Tucker, I would like to provide you with information on the manuscript preparation and submission process.

Manuscript preparation and submission: The submitted manuscript should be a full review of the presentation topic, with balanced consideration of the literature and state of the science. The manuscript should not be limited to a summary of the meeting presentation, and it should not include unpublished original data. Manuscripts should be prepared in accordance with the most recent "Information for Authors" found online at http://advances.nutrition.org/site/misc/ifora.xhtml. *Advances in Nutrition* is limited in the number of pages that can be published each year and article length is a consideration in the editorial process. Papers should be limited to 8 – 10 published pages with a word count ≤ 7500 words. Word count includes abstract, text, figure legends, acknowledgments, and references. All manuscripts must be submitted using the *Advances in Nutrition* online manuscript submission site (http://submit.an.nutrition.org/).

Please note that if there is topical overlap between your presentation and that of another speaker, you could consider authoring a manuscript in collaboration with the other speaker.

Authors will not be assessed submission fees, and authors of accepted manuscripts will not be assessed page charges or color reproduction fees. Authors will be assessed fees for discretionary author alterations and for reprints, if ordered.

Manuscript review and acceptance: Manuscripts submitted for publication *to Advances in Nutrition* are reviewed with the same stringency and criteria as all other manuscripts submitted to the Journal. This invitation does not give or imply a guarantee of eventual acceptance, which will be determined by the outcome of the review and editorial process. The Editor retains the right to return a manuscript to the author for revision and may refuse to accept any manuscript not considered suitable for publication in *Advances*

Submission deadlines: Individual presenters who wish to submit a review manuscript based on their presentation should submit the manuscript as soon as possible, but no later than April 6, 2015, four months after the conclusion of the meeting.

Kindly confirm your interest in submitting a manuscript to *Advances in Nutrition* by Friday, December 12, 2014.

Please do not hesitate to contact me at any time if you would like additional information about the manuscript preparation or submission process.

Kind regards,

Katie Dunn
Journal Manager
Advances in Nutrition
Journal Website: http://advances.nutrition.org/
Email: advances@nutrition.org
Telephone: 301-634-7115