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Mikko Kuokkanen

Molecular Genetics of Lactase Deficiencies

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Department of Molecular Medicine
National Public Health Institute Helsinki, Finland
and
Department of Medical Genetics, University of Helsinki, Finland

Helsinki 2006

Mikko Kuokkanen

MOLECULAR GENETICS OF LACTASE DEFICIENCIES

ACADEMIC DISSERTATION

To be presented with the permission of the Medical Faculty, University of Helsinki, for public examination in Lecture Hall 3 of Biomedicum Helsinki, Haartmaninkatu 8,on December 5th, at 12 noon.

Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland

and

Department of Medical Genetics, University of Helsinki, Finland

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The only solution.

Isn't it amazing?

Jim Morrison, Shaman’s Blues (1969)

To my family

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ABSTRACT

Congenital lactase deficiency (CLD) (MIM 223000) is a rare autosomal recessive gastrointestinal disorder characterized by watery diarrhea in infants fed with breast milk or other lactose-containing formulas. The CLD locus was previously assigned by linkage and linkage disequilibrium analyses on 2q21 in 19 Finnish families. In this study, the molecular background of this disorder is reported. The CLD locus was refined in 32 CLD patients in 24 families by using microsatellite and single nucleotide polymorphism (SNP) haplotypes. Mutation analyses were performed by direct sequencing. We identified 5 distinct mutations in the lactase (*LCT*) gene, encoding the enzyme that hydrolyzes lactose in the intestinal lumen. Twenty-seven patients out of thirty-two (84%) were homozygous for a nonsense mutation, c.4170T→A (Y1390X, designated as Fin_{major}). In addition, four rare mutations were detected two of which, a four-nucleotide deletion (c.4998_5001delTGAG) and a two-nucleotide deletion (c.653_654delCT), predicted a frameshift and protein truncation at S1666fsX1722 and S218fsX224 of the 1927 amino acid polypeptide, respectively. Two point mutations, c.804G→C and c.4087G→A, would result in amino acid substitutions Q268H and G1363S, respectively. Five patients were compound heterozygous carrying the Fin_{major} mutation and one of the four minor mutations. These findings facilitate genetic testing of CLD in clinical practice and enable genetic counseling. The present data also provide the basis for detailed characterization of the molecular pathogenesis of this disorder.

Adult-type hypolactasia (MIM 223100) (lactase non-persistence, lactose intolerance) is an autosomal recessive gastrointestinal condition that is a result of a decline in the activity of lactase in the intestinal lumen after weaning. Adult-type hypolactasia is considered to be a normal phenomenon among mammals and symptoms are remarkably milder than experienced in CLD. Recently, a variant C/T_{-13910} was shown to associate with the adult-type hypolactasia trait, locating 13.9 kb upstream of the *LCT* gene (Enattah et al. 2002). In this study, the functional significance of the C/T_{-13910} variant was determined by studying the *LCT* mRNA levels in intestinal biopsy samples in children and adults with different genotypes. Intestinal biopsy samples were taken from 15 children or adolescents and from 52 adults with abdominal complaints. The expression of *LCT* mRNA was demonstrated in patients

heterozygous for the C/T$_{-13910}$ variant and an informative expressed SNP located in the coding region of *LCT*. RT-PCR followed by solid-phase minisequencing was applied to determine the relative expression levels of the *LCT* alleles using an informative SNP located in exon 1. In children, the C$_{-13910}$ allele was observed to be downregulated after five years of age in parallel with lactase enzyme activity. The expression of the *LCT* mRNA in the intestinal mucosa in individuals with the T$_{-13910}$ A$_{-22018}$ alleles was 11.5 times higher than that found in individuals with the C$_{-13910}$, G$_{-22018}$ alleles. These findings suggest that the C/T$_{-13910}$ associated with adult-type hypolactasia is associated with the transcriptional regulation of the *LCT* gene. The presence of the T$_{-13910}$ A$_{-22018}$ allele also showed significant elevation lactase activity.

Galactose, the hydrolysing product of the milk sugar lactose, has been hypothesized to be poisonous to ovarian epithelial cells. Hence, consumption of dairy products and lactase persistence has been proposed to be a risk factor for ovarian carcinoma. To investigate whether lactase persistence is related to the risk of ovarian carcinoma the C/T$_{-13910}$ genotype was determined in a cohort of 782 women with ovarian carcinoma. The C/T$_{-13910}$ genotype was determined by the solid-phase minisequencing method from 327 Finnish, 303 Polish, 152 Swedish patients and 938 Finnish, 296 Polish and 97 Swedish healthy subjects serving as controls. Lactase persistence did not associate significantly with the risk for ovarian carcinoma in the Finnish (OR 0.77, 95% CI, 0.57-1.05, p=0.097), in the Polish (OR 0.95, 95% CI, 0.68-1.33, p=0.75), or in the Swedish populations (OR 1.63, 95% CI, 0.65-4.08, p=0.29). The findings do not support the hypothesis that lactase persistence increases the risk for ovarian carcinoma.

Keywords: *LCT*, CLD, adult-type hypolactasia, lactase persistence/non-persistence, ovarian carcinoma, C/T$_{-13910}$, single nucleotide polymorphism, solid-phase minisequencing

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TIIVISTELMÄ

Synnynnäinen laktaasin puutos (congenital lactase deficiency, CLD [MIM 223000]) on vakava peittyvästi periytyvä suolistosairaus. Potilailla on tyypillistä runsas vetinen ripuli, joka ilmenee maitoruokinnan alettua. Ripuli johtaa ravintoaineiden imeytymishäiriöön ja kasvun hidastumiseen. Potilaiden laktaasiaktiivisuus on havaittu ohutsuolessa hyvin alhaiseksi. Vaikea ripuli johtuu nimenomaan hydrolysoimattoman laktoosin kertymisestä suolistoon aiheuttaen osmoosin, vaikean kuivumistilan, asidoosin ja painon menetyksen syntymäpainon alapuolelle. Laktoositon ruokavalio poistaa oireet ja palauttaa kehityksen normaaliksi. CLD-vauvoja syntyy Suomessa yksi vuodessa (1:60000). Se kuuluu yhdessä 36 muun harvinaisen Suomessa esiintyvän sairauden kanssa ns. suomalaiseen tautiperintöön.

Tutkimuksen tarkoituksena oli tunnistaa CLD:lle altistavat geneettiset tekijät geenien kartoitus- ja sekvensointimenetelmien avulla. Tutkimuksessa analysoitiin 32 CLD-potilasta 24 eri perheestä. Löysimme viisi CLD:lle altistavaa mutaatiota laktaasigeenistä (*LCT*), joista Y1390X-mutaation havaittiin olevan yleisin ns. $\text{Fin}_{\text{major}}$-mutaatio. Löydökset mahdollistavat sairauden perinnöllisen testauksen ja neuvonnan.

Lapsuuden jälkeen tai nuorella aikuisiällä havaittu primääri maitosokerin imeytymishäiriö (MIM 223100) (tunnettu myös laktoosi-intoleranssina) on yleinen laktaasin aktiivisuuden alenemisesta johtuva ilmiö. Laktaasiaktiivisuus alenee suolistossa 5-10% lapsuudesta havaitusta ja laktoosi ruokavaliossa aiheuttaa suolistoperäisiä oireita. Laktaasin puutos on nisäkkäille luonnollinen ilmiö, näin lapsi vierotetaan rinnasta käyttämään kiinteätä ravintoa. Kuitenkin joillain ihmisillä korkea laktaasiaktivisuus säilyy koko elinajan. Kyseiseen ilmiasuun on havaittu

liittyvän yhden emäksen muutos $C \rightarrow T_{-13910}$ noin 14 kiloemästä laktaasigeenistä ylävirtaan. Muutos sijaitsee *MCM6*- (minichromosome maintenance deficient 6) geenin intronissa 13 kromosomissa 2q21. T_{-13910}-muutoksen perineet henkilöt säilyttävät laktaasiaktiivisuuden ja kyvyn pilkkoa laktoosia.

Tämän tutkimuksen tarkoituksena oli määrittää C/T_{-13910} emäsmuutoksen vaikutus laktaasigeenin ilmentymiseen lähetti-RNA tasolla lapsilla ja aikuisilla. Laktaasigeenin ilmentymistä tutkittiin geenispesifisellä minisekvensointimenetelmällä pohjukaissuolesta otetuista näytteistä. Havaitsimme, että laktaasia säädellään transkriptiotasolla, C_{-13910}-muutos sallii laktaasigeenin lähetti-RNA:n laskun. Ilmiö oli havaittavissa lapsilla viiden ikävuoden jälkeen. Aikuisilla T_{-13910}-emäsmuutos vastasi 92% havaitusta laktaasigeenin lähetti-RNA:n ilmentymisestä, joka näkyi myös korkeana laktaasiaktiivisuutena.

Galaktoosi on glukoosin ohella laktoosin hajoamistuote. Galaktoosin on havaittu eläinkokeissa olevan myrkyllinen munasarjojen epiteelisoluille ja sen on epäilty aiheuttavan munasarjasyöpää. Tästä syystä korkean laktaasiaktiivisuuden ja maitotuotteiden runsaan nauttimisen on oletettu nostavan munasarjasyövän riskiä. Tässä tutkimuksessa määritettiin 782 suomalaisen, puolalaisen ja ruotsalaisen munasarjasyöpäpotilaan ja 1331 verrokkinäytteen C/T_{-13910}-genotyyppi tutkiaksemme onko korkealla laktaasiaktiivisuudella yhteyttä syövän syntyyn. Genotyypillä ei havaittu olevan vaikutusta syöpäriskiin tutkituissa väestöryhmissä. Jatkotutkimuksella pyritään selvittämään maitotuotteiden kulutuksen merkitystä munasarjasyöpään eri C/T_{-13910} genotyypeillä.

Avainsanat: synnynnäinen laktaasin puutos, geenikartoitus, suomalainen tautiperintö, laktoosin imeytymishäiriö, C/T_{-13910}, minisekvensointi, munasarjasyöpä, galaktoosi

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ABBREVIATIONS

χ^2	chi-square test
λ	proportion of excess of alleles in chromosomes carrying the disease allele
θ	recombination fraction
Caco-2 cell	human intestinal adenocarcinoma cell
cDNA	complementary DNA
Cdx	caudal-related protein
CEPH	Centre d'Etude du Polymorphisme Humain
CLD	congenital lactase deficiency
cM	centiMorgan
cSNP	coding single nucleotide polymorphism
DARS	aspartyl-tRNA synthetase
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EST	expressed sequence tag
FISH	fluorescence *in situ* hybridization
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HNF	hepatocyte nuclear factor
HRR-LRT	haplotype relative risk likelihood-ratio
in situ	on the spot
in vitro	in test tube
L	likelihood
LCT	lactase

LD	linkage disequilibrium
LOD, Z	logarithm of odds
L/S	ratio of lactase to sucrase
LTTE	lactose tolerance test with ethanol
MCM6	minichromosome maintenance deficient 6
MIM	Mendelian Inheritance in Man
mRNA	messenger RNA
NMD	Non-sense mediated mRNA decay
Oct	octamer-binding transcription factor
OMIM	Online Mendelian Inheritance in Man
PCR	polymerase chain reaction
PTH	parathyroid hormone
q	long arm of chromosome
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcriptase
SCCA	squamous cell carcinoma antigen
SNP	single nucleotide polymorphism
STR	short tandem repeat
tRNA	transfer RNA

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Kuokkanen M, Kokkonen J, Enattah NS, Ylisaukko-oja T, Komu H, Varilo T, Peltonen L, Savilahti E, & Järvelä I. (2006) Mutations in the Translated Region of the Lactase Gene (*LCT*) Underlie Congenital Lactase Deficiency. Am J Hum Genet. 78:339-44

II Rasinperä H, Kuokkanen M, Enattah NS, Kolho KL, Savilahti E, Orpana A & Järvelä I. (2005): Transcriptional downregulation of the lactase (*LCT*) gene during childhood. Gut. 54:1660-1

III Kuokkanen M, Enattah NS, Oksanen A, Savilahti E, Orpana A & Järvelä I (2003): Transcriptional regulation of the lactase-phlorizin hydrolase gene by polymorphisms associated with adult-type hypolactasia. Gut 52:647-652.

IV Kuokkanen M, Butzow R, Rasinperä H, Medrek K, Nilbert M, Malander S, Lubinski J & Järvelä I. (2005): Lactase persistence and ovarian carcinoma risk in Finland, Poland and Sweden. Int J Cancer. 117:90-94

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Publications II and III has previously appeared in theses by Heli Rasinperä (2006) and Nabil Sabri Enattah (2005), respectively.

1 INTRODUCTION

Congenital lactase deficiency (CLD) is a severe gastrointestinal disorder in newborns. It is characterized by watery diarrhea during the first days of life in infants who are fed lactose-containing milk. Diarrhea is caused by diminished lactase activity in the intestinal mucosa. CLD is a rare autosomal recessively inherited disorder and belongs to the group of 36 rare diseases found in Finland (Norio et al. 1973; Savilahti et al. 1983; Norio 2000; Norio 2003c). Holzel and colleagues (1959) discovered the first patients in 1959. Adult-type hypolactasia (also known as lactase non-persistence, primary lactose malabsorption) is a common gastrointestinal condition whose recessive inheritance was shown in 1973 (Sahi et al. 1973). It is caused by developmental downregulation of lactase (*LCT*) resulting in unhydrolysed lactose in the intestinal mucosa. Clinical symptoms (lactose intolerance) such as diarrhea are mainly due to osmosis (Auricchio et al. 1963; Dahlqvist et al. 1963). Adult-type hypolactasia represents a normal physiological condition after weaning (Simoons 1970). However, the majority of northern Europeans and some nomadic population in Africa and Arabia have the ability to maintain lactase activity and digest lactose throughout life (lactase persistence) (Swallow and Hollox 2000). Adult-type hypolactasia has shown to associate with the C/T_{-13910} variant, which is located 13.9 kilobases (kb) upstream of the *LCT* gene. The C/C_{-13910} genotype is associated with lactase non-persistence whereas C/T_{-13910} and T/T_{-13910} genotypes are associated with lactase persistence.

Galactose, the hydrolyzing product of the milk sugar lactose, has been hypothesized to be toxic to ovarian epithelial cells and consumption of dairy products with lactase persistence has been suggested to be a risk factor for ovarian carcinoma. Ovarian carcinoma is the fourth most common cause of cancer death in women. The cause and pathogenesis of this disease has remained obscure.

In this thesis, linkage disequilibrium (LD) and haplotype mapping was applied to refine the CLD locus and direct sequencing to identify mutations underlying CLD. In addition, the functional effect of the variant C/T_{-13910} was studied on the mRNA level of the *LCT* gene in children and adults using the solid-phase minisequencing method. In order to assess whether lactase persistence is a risk factor for ovarian carcinoma we have determined the C/T_{-13910} variant in Finnish, Polish and Swedish women with ovarian carcinoma and defined its relation to their corresponding control populations.

2 REVIEW OF THE LITERATURE

2.1 The Finnish disease heritage

The exceptional pattern of rare hereditary disorders in Finland, the Finnish disease heritage, was introduced by Doctors Norio, Nevanlinna and Perheentupa in 1973 (Norio et al. 1973). The very first of these distinctive disorders the Finnish type congenital nephrotic syndrome was described in 1956 (Hallman et al. 1956). Its mode of inheritance was traced back ten years later from the very carefully maintained records of church books reaching back to the 16th century (Norio 1966). Records of church books and land tax registers have been the keys to exploring Finnish hereditary disorders. There are 36 disorders which belong to the Finnish disease heritage, 32 of which are autosomal recessive, two X-chromosomal and two dominantly inherited (Table 1). The incidence of these disorders is in the range of 1:10000-1:100000. The higher prevalence of certain disorders in Finland and on the other hand the lack of disorders that are prevalent elsewhere in the world have inspired researchers to investigate the origin of Finns and their genes (Markkanen et al. 1987; Norio 2000; Norio 2003a). Disorders of the Finnish disease heritage are introduced in Table 1.

Table 1. Disorders of the Finnish disease heritage and their defective proteins or loci in alphabetical order

Disease [OMIM number]	Defective gene/protein or locus	Reference
Amyloidosis V [105120] **(dominant)**	Gelsonin (GSN)	Levy et al. 1990, Maury et al. 1990
Aspartylglucosaminuria (AGU) [208400]	Aspartylglucosaminidase (AGA)	Ikonen at al. 1991
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) [240300]	Autoimmune regulator (AIRE)	Nagamine et al. 1997, The Finnish-German APECED Consortium 1997
Batten disease [204200]	CLN3	The International Batten Disease Consortium 1995
Cartilage-hair hypoplasia (CHH) [250250]	RNA component of Rnase MRP (RMRP)	Ridanpää et al. 2001
Choroidemia (CHM) [303100] **(X-linked)**	Rab escort protein 1 (REP1)	Sankila et al. 1992
Cohen syndrome (COH1) [216550]	COH1	Kolehmainen et al. 2003
Congenital chloride diarrhea (CCD) [214700]	Solute carrier family 26, member 3 (SLC26A3)	Höglund et al. 1996
Congenital lactase deficiency (CLD) [223000]	LCT	Study I
Congenital nephrosis (CNF) [256300]	Nephrin	Kestilä et al. 1998
Cornea plana congenita (CNA2) [217300]	Keratocan (KERA)	Pellegata et al. 2000
Diastrophic dysplasia (DTD) [222600]	Solute carrier family 26, member 2 (SLC26A2)	Hästbacka et al. 1994

Free sialic acid storage disease (Salla disease) [604369]	Solute carrier family 17, member 5 (SLC17A5)	Verheijen et al. 1999
Growth retardation, aminoaciduria, cholestasis, iron overload lactacidosis and early death (GRACILE) [603358]	BCS1-like (BCS1L)	Visapää et al. 200
Gyrate atropy (GA) [258870]	Ornithine aminotransferase (OAT)	Mitchell et al. 1989
Hydrolethalus syndrome (HLS) [236680]	HYLS 1	Mee et al. 2005
Hypergonadotrophic ovarian dysgenesis (ODG1) [233300]	FSH receptor (FSHR)	Aittomäki et al. 1995
Infantile neuronal ceroid-lipofuscinosis (INCL) [256730]	Palmitoyl protein thioesterase 1	Vesa et al. 1995
Infantile onset spinocerebellar ataxia (IOSCA) [271245]	Twinkle and Twinky	Nikali et al. 2005
Lethal arthrogyposis with anterior horn cell disease	unknown	Clinical characterization by Vuopala et al. 1995
Lethal congenital contracture syndrome (LCCS) [253310]	9q34	Mäkelä-Bengs et al. 1998
Lysinuric protein intolerance (LPI) [222700]	Solute carrier family 7, member 7 (SLC7A7)	Borsani et al. 1999, Torrents et al. 1999
Meckel syndrome (MKS1) [249000]	MKS1	Kyttälä et al. 2006
Megaloblastic anemia 1 [261100]	Cubilin (CUBN)	Aminoff et al. 1999
Mulibrey nanism [253250]	TRIM37	Avela et al. 2000
Muscle-eye-brain disease (MEB) [253280]	POMGnT1	Diesen et al. 2004
Nonketotic hyperglycinemia (NKH) [605899]	Glycine decarboxylase (GLDC)	Kure et al. 1992

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLO-SL) [221770]	TYRO protein tyrosine kinase-binding protein (TYROBP) and TREM2	Paloneva et al. 2000, Paloneva et al. 2002
Progressive encephalopathy with edema, hypsarrhythmia and optic atrophy (PEHO syndrome) [260565]	unknown	Clinical characterization by Salonen et al. 1991
Progressive epilepsy with mental retardation (EPMR) [600143]	CLN8	Ranta et al. 1999
Progressive myoclonus epilepsy (EPM1) [254800]	Cystatin B (CSTB)	Pennacchio et al. 1996; Virtaneva et al. 1997
RAPADILINO syndrome [266280]	RECQL4	Siitonen et al. 2003
Retinoschisis [312700] **(X-linked)**	Retinoschisin	The Retinoschisis Consortium 1998
Tibial muscle dystrophy (TMD) [600334] **(dominant)**	Titin (TTN)	Hackman et al. 2002
Usher syndrome type III [276902]	USH3A	Joensuu et al. 2001
Variant late infantile neuronal ceroid-lipofuscinosis (vLINCL) [256731]	CLN5	Savukoski et al. 1998

Thirty-two disorders are recessively inherited, two are dominantly inherited and two are X-linked traits (in **bold** text). The table is based on Norio (2000) and Norio (2003c).

2.1.1 Causes of the unique gene pool

2.1.1.1 The settlement history of Finland

The predominant hypothesis is that Finland has been inhabited continuously from the last glacial period approximately 10000 years ago (Nunez 1987; Norio 2003b). The oldest archaeological discovery from southern Finland is circa 9000 years old

whereas the first signs of settlement date from 8000 years ago (Huurre 1992; Jutikkala and Pirinen 1996). It cannot be known for sure who the first settlers were. They could have been inhabitants, despite the arctic circumstances, living on the coast of Scandinavia from the glacial period or/and immigrants who returned from the south as the ice melted. However, some of these first settlers are probably the ancestors of the Saami and some of them adopted agriculture and merged with the growing southern settler groups. In prehistoric Scandinavia times the warm Gulf stream offered bearable living conditions for a small population whose source of livelihood was based on hunting, fishing, and gathering (Norio 2000; Norio 2003b). Nowadays, there are only 60000 Saami living in the northern regions of Finland, Sweden, Norway and the Russian Kola Peninsula of these 6000 individuals live in Finland. The climate and source of livelihood were not conducive to expansion of population, the size has been estimated to have been constant throughout history (Lehtivirta and Seurujärvi-Kari 1991). Still warming climate forced the Saami to retreat gradually to the north away from growing settlers groups who had adopted agriculture (Eriksson 1973; Lehtivirta and Seurujärvi-Kari 1991). These settler groups carried the genes of today's Finns. Where did the farmers come from? The dual theory of Finland's inhabitation by Eriksson (1973) and Norio (1981) suggests there was an early migration of eastern Uralic speakers ~4000 years ago and a later migration from the south ~2000 years ago (Figure 1). Kittles and his colleagues's (1998) Y chromosome haplotype study supported this theory as they observed two different male lineages. Mitochondrial and nuclear DNA analyses show that the majority of Finnish genes are of central European origin (Cavalli-Sforza and Piazza 1993; Sajantila et al. 1995; Sajantila and Pääbo 1995; Lahermo et al. 1996; Laan and Pääbo 1997; Torroni et al. 1998). The Saami and other Europeans including Finns have been suggested to have a divergent population history (Cavalli-Sforza and Piazza 1993; Sajantila et al. 1995; Sajantila and Pääbo 1995; Lahermo et al. 1996). A recent mitochondrial and Y chromosomal study suggests that most likely the Saami have European origin after all. The large genetic difference between the

Saami and other Europeans is explained by the Saami representing a narrow subset of Europeans (Tambets et al. 2004). Since the last glacial period all migrations from south, east and also small ones from west shaped the Finnish population genetically, linguistically and culturally but kept as a relatively small founder population.

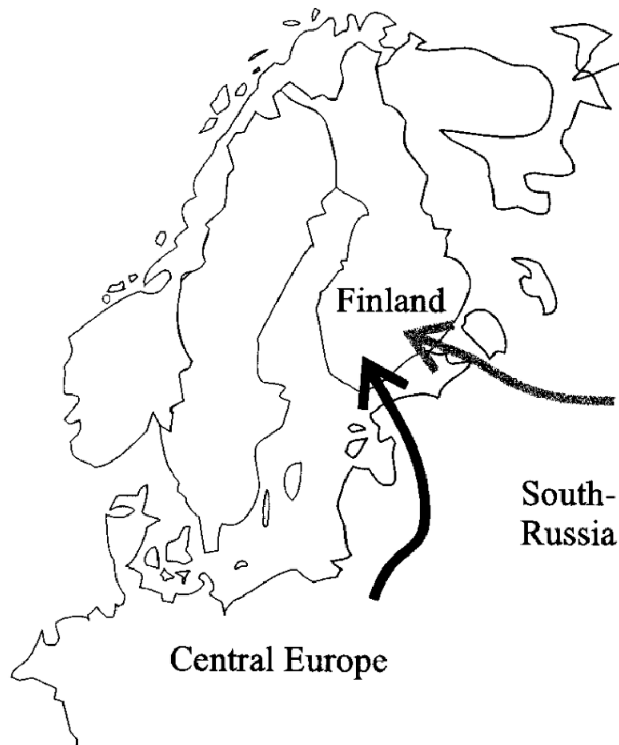


Figure 1. The main two migration waves came to the area of Finland from south and east. Adapted from Varilo (1999).

2.1.1.2 The internal migration in the 16th century

In the 16th century the Finnish population had grown to 250 000 and inhabitation who were focused in the coastal regions (Figure 2). Sweden acquired Denmark after the Liberation War in 1521-1523 and Gustavus of Vasa (1523-1560) was crowned

monarch of a new independent state Sweden-Finland. King Gustavus of Vasa was interested in his kingdom and made administrative reforms, which included increasing taxation and district court sessions. The superpower politics of King Gustavus of Vasa favoured the inhabitation of wildernesses to enlarge the country's borders. Farming were encouraged by lower taxation for farmers; also the growth of the population led to pressure to cultivate more land. The majority of inhabitants originated from the area of southeastern Finland called South Savo (Figure 2). Within two centuries nearly the whole area of Finland was inhabited and the population had increased to 400000. There was a great famine in 1696-1698 which decreased the population to 250000 but from the 1700s the Finnish population has grown pretty quickly to its present figure of 5300000 e.g. (Jokipii 1992; Jokipii and Rikkinen 1992; Jutikkala and Pirinen 1996; Varilo 1999; Norio 2003a).

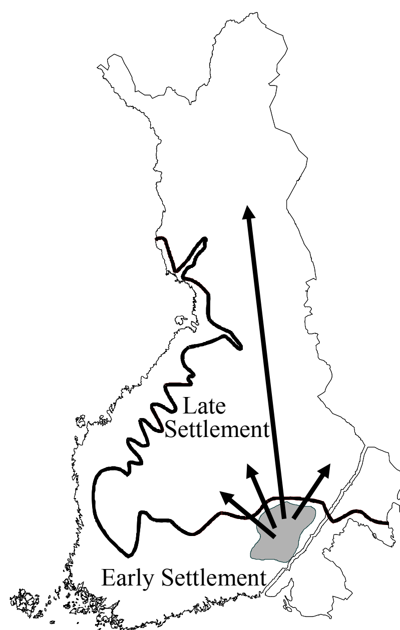


Figure 2. The internal migration movement led to regional subisolates in the Finland area in the 16th century. The map is modified from Varilo (1999).

The small number of original ancestors both due to genetic drift and regional subisolates, a rapid increase in population and linguistic and geographical isolation have strongly shaped the Finnish population and created our unique gene pool. Our carefully maintained church books, land registers and history books have given us tools to study our origin and rare diseases (Norio et al. 1973; Norio 2000).

2.2 Lactase

Lactase (*LCT*), also known as lactase-phlorizin hydrolase, belongs to the β-galactosidase family having both lactase (EC 3.2.1.108) and phlorizin hydrolase activity (EC 3.2.1.62). *LCT* is exclusively expressed in the small intestine and it is responsible for hydrolysing lactose (Figure 3) to glucose and galactose. Lactose is a major disaccharide found in milk synthesized by lactose synthetase in the mammary gland. Human milk has the highest lactose content (7%) e.g. cow milk contains 4.8% of lactose. Phlorizin hydrolase is responsible for hydrolysing aryl- and alkyl-β-glycosides such as phlorizin and galactosyl- and glycosyl-β-ceramides (Sahi 1994b). Lactase activity is delimited in mammals whereas phlorizin hydrolase activity has been detected in vertebrates. The natural substrate of phlorizin hydrolase has been thought to be glycosyl ceramides that inhere in the diet of most vertebrates and are also found in milk (Leese and Semenza 1973; Skovbjerg et al. 1981). Both catalytic activities are located on a single polypeptide chain (Wacker et al. 1992).

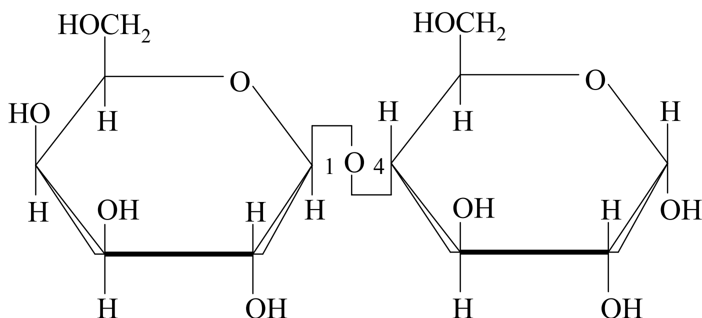


Figure 3. Lactose the milk sugar. In lactose, a glucose unit and a galactose unit are joined by an β-1, 4 glycosidic linkage.

2.2.1 Structure

The *LCT* gene reaches a genomic size of 50 kb and it is composed of 17 exons. There is one kb promotor region preceding *LCT* (Boll et al. 1991). The size of messenger RNA (mRNA) is 6274 bases and the primary translation product (pre-pro-LCT) is 1927 amino acids (Figure 3). Pre-pro-LCT consists of an N-terminal signal sequence, pre-domain (19 amino acids), a pro-domain size of 849 amino acids, an extracellular domain of 1014 amino acids, a hydrophobic transmembrane anchor domain (19 amino acids), and a short C-terminal cytosolic domain of 26 amino acids. Pro-LCT contains four internal repeats of which the pro-domain includes repeats I-II and an extracellular domain includes repeats III-IV (Figure 4). Phlorizin hydrolase activity locates in repeat III and lactase activity locates in repeat IV (Neele et al. 1995; Zecca et al. 1998; Arribas et al. 2000). These four repeats contain 38-55% identical amino acids. Mantei and colleagues (1988) suggest that repeats III-IV are results of duplication of the I-II repeats of one ancestor gene. Sequence similarities of the I-IV regions to β-glycosidases of archaebacteria, eubacteria, and fungi support this hypothesis and thus, lactase belongs to the β-glucosidase and β-galactosidase superfamily (Naim 2001).

2.2.2 Biosynthesis

Mature lactase is anchored to the intestinal membrane by a hydrophobic region near its carboxy terminus and the catalytic sites of the enzyme are located in the lumen of the intestine (Mantei et al. 1988). How is a lactase polypeptide processed to its active form? Intestinal epithelial cells synthesize lactase as a single chain pre-pro-LCT precursor polypeptide that is translocated over the endoplasmic reticulum (ER) guided by a signal sequence (Figure 4). The signal sequence is removed during the process resulting in pro-LCT with a molecular weight of 215 kiloDalton (kDa) (Naim and Naim 1996). Even if the pro-region of LCT (LCTα) does not have enzymatic activity (Naim 1995) it is has been shown to function as an intramolecular chaperone essential for the folding of pro-LCT (Oberholzer et al. 1993; Naim et al. 1994; Jacob et al. 2002). In ER, pro-LCT is glycosylated by mannose rich N-linked oligosaccharides. Glycosylated pro-LCT forms a homodimer that is further transferred to the Golgi apparatus. The transmembrane anchor domain and the cytosolic domain participate directly in the dimerization and apical sorting of lactase. The O-linked sugars of the pro-LCT dimer are glycosylated and N-linked sugars are further processed in cis-Golgi resulting in a glycoprotein with a molecular weight of 230 kDa. Glycosylation and dimerization of pro-LCT is required for efficient intracellular transport and enzyme activity (Naim et al. 1991; Naim and Lentze 1992; Naim 1994; Naim and Naim 1996; Panzer et al. 1998; Jacob et al. 2000). Pro-LCT undergoes two proteolytic cleavage steps before it finally becomes mature lactase. The first cleavage takes place intracellulary and removes the large LCTα at R734/L735 resulting in a membrane bound $LCT\beta_{initial}$ (L735-Y1927). $LCT\beta_{initial}$ is targeted to the intestine brush border membrane where it is cleaved by trypsin at R868/A869 leading to a 160 kDa mature lactase, $LCT\beta_{final}$ (Figure 4) (Jacob et al. 1996; Wüthrich et al. 1996).

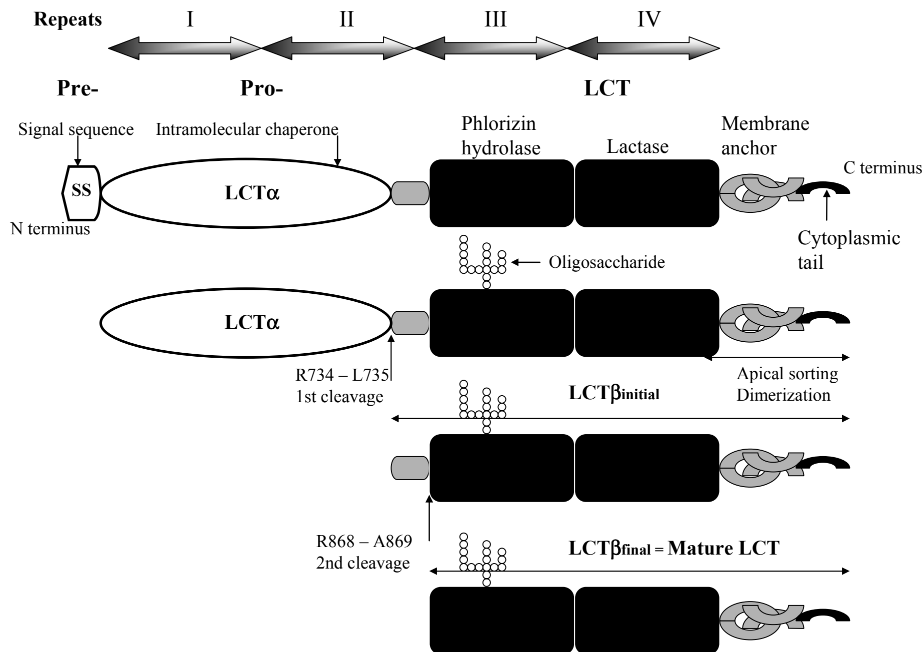


Figure 4. The structure and biosynthesis of lactase. Lactase is synthesized as a pre-pro-LCT precursor. The pre-pro-LCT contains a cleavable signal sequence that translocates the polypeptide over the endoplasmic reticulum (ER) (von Heijne 1986; Mantei et al. 1988). The pro-lactase region consists of four homologous repeats (I-IV). Repeats I and II are located in the pro-domain while repeats III-IV are found in mature lactase (Mantei et al. 1988). Later in the ER, the signal sequence is cut off yielding pro-LCT that is N-glycosylated and forms homodimers (Naim et al. 1991; Naim and Lentze 1992; Naim 1994; Naim and Naim 1996; Panzer et al. 1998; Jacob et al. 2000). The region involved in apical sorting and dimerization is indicated. The pro-region, LCTα, is shown to function as an intramolecular chaperone for folding of pro-LCT (Oberholzer et al. 1993; Naim et al. 1994; Jacob et al. 2002). The pro-LCT is transferred to the Golgi apparatus and O-glycosylated. The pro-LCT is further processed by two proteolytic cleavages: an intracellular cleavage that occurs between R734 and L735 produces LCT$\beta_{initial}$ and a cleavage in the intestinal lumen between R868-A869 generates LCTβ_{final} the mature enzyme (Jacob et al. 1996; Wüthrich et al. 1996). The figure is based on Naim (2001) and Troelsen (2005).

2.2.3 Regulation

Milk is the major nutrient consumed by newborn mammals. As lactose is the main carbohydrate and the most important source of energy in milk, it is necessary for mammals to be able to hydrolyse lactose after birth. *LCT* is expressed only by small intestinal enterocytes located at the crypt-villus junction and on the villus. Although, lactase activity is required from birth in mammals the timing of high *LCT* expression differs between rodents and humans. Humans have high *LCT* expression from birth whereas in mouse lactase expression reaches its maximum level 3 days after birth. This has been thought to be due to the intestinal immaturity of rodents. Fully developed crypts are not detected until 2-3 days after birth in mice. This finding has made *LCT* an ideal marker for fully differentiated enterocytes (Klein 1989; Freeman et al. 1993; Freund et al. 1995; Troelsen 2005).

2.2.3.1 Proximal LCT promoter

The proximal LCT promoter, a 150-basepair fragment upstream of the transcription initiation site (-150), is conserved in mouse, rat, rabbit, pig and human. Promoter analyses have revealed three transcription factor binding sites (CE1a, CE2c and GATA-site) so called cis-elements in this fragment (Figure 5) (Troelsen et al. 1992; Fitzgerald et al. 1998; Spodsberg et al. 1999). The functional role of transcription factors in these binding sites has been studied in transfection studies of promoter-reporter gene constructs and in DNA-protein and protein-protein interaction analyses using human intestinal adenocarcinoma (Caco-2) cells.

It has been shown that the caudal-related homeobox protein (Cdx-2) and the homeobox protein HOXC11 bind to CE1a (Troelsen et al. 1997; Mitchelmore et al. 1998; van Wering et al. 2002b). Cdx-2 is a significant factor for intestinal transcription and maintenance (Chawengsaksophak et al. 1997; Freund et al. 1998) and it is capable of activating pig (Troelsen et al. 1997) rat (Fang et al. 2000;

Krasinski et al. 2001) and human LCT promoters (Krasinski et al. 2001). Cdx-2 was also detected to bind to a site overlapping the *LCT* TATA-box. However, it remained unknown whether Cdx-2 binds directly to the site or through protein-protein interactions with a transcription initiation complex (van Wering et al. 2002b). The significance of this observation is to be clarified. HOXC11 is expressed in human fetal intestines but it could not be detected after birth suggesting a role in early intestinal development (Mitchelmore et al. 1998). Additionally, there are unidentified factors which repress *LCT* expression through the CE1a-site (Troelsen et al. 1997; van Wering et al. 2002b). Repression of *LCT* transcription by those factors was suggested to take place in cells that do not express Cdx-2 (van Wering et al. 2002b). This would indicate a tissue-specific LCT promoter activation by Cdx-2.

CE2c has been shown to be crucial for the LCT promoter activity. Mutations of the CE2c-site remarkably decrease the LCT promoter activity in pigs (Spodsberg et al. 1999) and in human (van Wering et al. 2002a). Hepatocyte nuclear factor-1α (HNF-1α) has been shown to bind to the CE2c-site (Mitchelmore et al. 1998; Spodsberg et al. 1999; Mitchelmore et al. 2000; Krasinski et al. 2001; van Wering et al. 2002a). HNF-1α has been shown to activate pig (Spodsberg et al. 1999) rat and human LCT promoters (Krasinski et al. 2001). Furthermore, HNF-1α and Cdx-2 has been shown to physically interact with and cooperatively activate pig (Mitchelmore et al. 2000) and human LCT promoters (Krasinski et al. 2001). HNF-1α was shown to be essential for *LCT* expression *in vivo*. *LCT* mRNA was reduced 95% in HNF-1α(-/-) mice compared with wild types (Bosse et al. 2006). HNF-1 transcription factors are expressed in a number of tissues such as intestine, liver, kidney, pancreas, stomach (Ott et al. 1991; Cereghini et al. 1992) and they have been shown to be involved in the activation of a number of intestinal genes (Bosse et al. 2006), genes of developmental appearance and embryonic regulation, as reviewed in Weber et al. (1996), and regulating the expression of genes that are expressed in the liver, kidney, and pancreas, reviewed in Pontoglio (2000).

GATA-4, -5 and -6 zinc finger transcription factor has been demonstrated to be capable of binding and activating individually the human (Fitzgerald et al. 1998; Krasinski et al. 2001), rat (Fang et al. 2001; Krasinski et al. 2001) LCT promoters. A study showed that GATA-4 is the key GATA-factor regulating lactase expression in mice (van Wering et al. 2004). GATA-4/HNF-1α and GATA-5/HNF-1α has been shown to physically interact with and synergistically activate mouse, rat and human LCT promoters (Krasinski et al. 2001; van Wering et al. 2002a; van Wering et al. 2004). GATA factors are expressed in a number of tissues. A subfamily of GATA-1, -2, and -3 is expressed in hematopoietic stem cells regulating differentiation-specific gene expression in T-lymphocytes, erythroid cells, and megakaryocytes, reviewed in Orkin (1998). A subfamily of GATA-4, -5, and -6 are expressed in a variety of mesoderm- and endoderm-derived tissues such as heart, liver, lung, gonad and intestine where they regulate tissue-specific gene expression, reviewed in Molkentin (2000).

The pancreatic duodenal homeobox-1 protein (Pdx-1) has been demonstrated to repress endogenous LCT promoter activity when Pdx-1 was overexpressed in Caco-2 cells. Pdx-1 is expressed the anterior duodenal region of the intestine (Guz et al. 1995) where *LCT* expression is repressed in adult mammals. A Pdx-1 candidate binding site TAAT was identified in the rat promoter but mutation analysis of the binding site failed to recover *LCT* expression suggesting a more complex regulation pattern (Wang et al. 2004). Pdx-1 binding sites have not been identified in human or pig LCT promoters (Troelsen 2005).

2.2.3.2 Distal regulatory elements

Even though, there is no doubt of the importance of the proximal LCT promoter in the regulation of the *LCT* gene it has a relatively weak effect on transcription in Caco-2 cells (Troelsen et al. 1992). There is evidence that distal regulatory elements, enhancers, are needed to complete the proximal promoter in order to obtain full *LCT* expression (Figure 5). This type of enhancer sequences was identified in the pig

locating around -850 of the lactase gene, this was necessary for high expression in Caco-2 cells (Spodsberg et al. 1999; Troelsen et al. 2003a). This enhancer region includes three binding sites CE2a, nt20 and CE2b of which CE2a is known to bind HNF-1α, the other two in binding factors are unidentified (Simon et al. 1995; Spodsberg et al. 1999; Troelsen et al. 2003a). The enhancer element is not conserved in human nor rat. Furthermore, a negative regulatory region CE3 has been identified in the pig and human promoter (Spodsberg et al. 1999). The Forkhead box (Fox) factors FREAC-2 and -3 have been shown to bind to this element in the pig, (Spodsberg et al. 1999) in human the same element binds intestinal nuclear factors (Hollox et al. 1999).

Experiments in transgenic mice have shown that distal regulatory sequences located at -2038-(+15) of the rat LCT promoter are important for tissue specificity, correct spatial and developmental expression (Krasinski et al. 1997; Lee et al. 2002; Wang et al. 2006). DNAse footprint analyses have revealed several Fox and a leucine zipper factor C/EBP in the rat LCT promoter (Figure 5). HNF-3 has been shown to activate the promoter whereas a negative regulatory region similar to the pig and human promoter repress the activity (Verhave et al. 2004). Even if distal regulatory regions are not conserved in pig, rat and human the same type of pattern does exist.

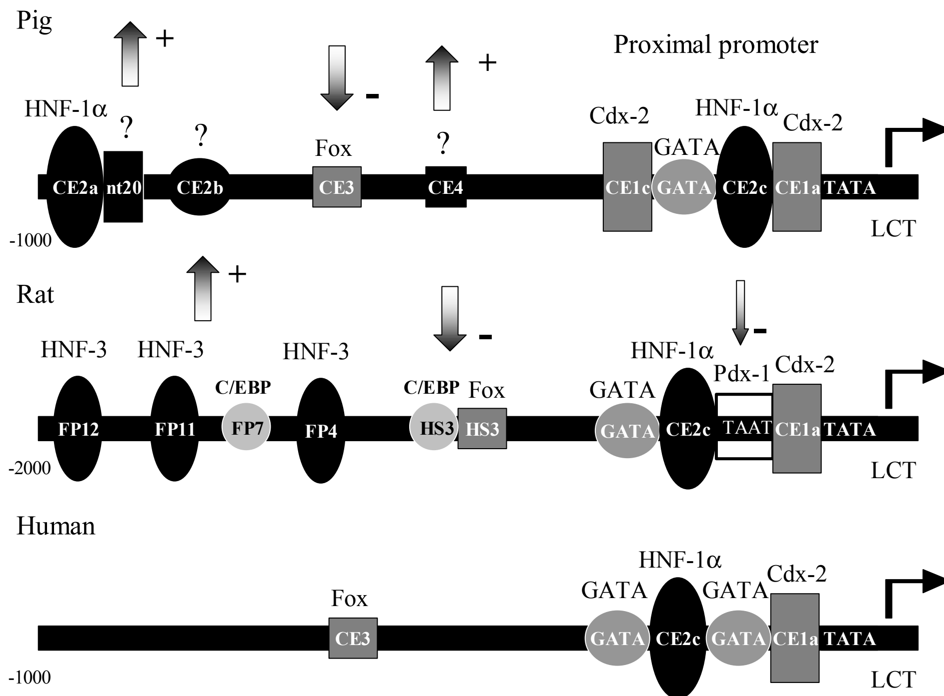


Figure 5. Proximal and distal regulatory elements of pig, rat and human LCT promoters. Arrows with + or – indicate the regulatory effect of cis-elements via proximal promoter on *LCT* transcription *in vitro*. Cis-elements are named as follows: CE (cis-element), HS (hypersensitive site) and FP (footprint). Transcription factors that bind the cis-elements are shown, question marks indicate unknown factors. The Figure is based on studies referred to on pages 26-29 and Troelsen (2005).

2.3 Congenital lactase deficiency

Congenital lactase deficiency (CLD [MIM 223000]) is an autosomal recessively inherited severe gastrointestinal disorder. Holzel et al. (1959) described the first patients in 1959. Launiala, Kuitunen, and Visakorpi (1966) discovered the absence of lactase activity in duodenal specimens of infants with explosive diarrhea after breastfeeding. In a clinical study on 16 patients Savilahti and colleagues (1983) gave the first evidence for a recessive mode of inheritance for CLD. So far, 50 patients in

42 families have been diagnosed in Finland (Savilahti et al. 1983; Study I) and several cases have been reported elsewhere in the world (Holzel 1967). CLD has an incidence of 1:60000 in the Finnish population (Savilahti E personal communication). The birthplaces of great-grandparents of 31 Finnish CLD families demonstrate that CLD mutation is emphasized in central Finland (Figure 6) (Järvelä et al. 1998). CLD is one of the 36 rare monogenic disorders enriched in Finland (Norio et al. 1973; Norio 2000; Norio 2003c).

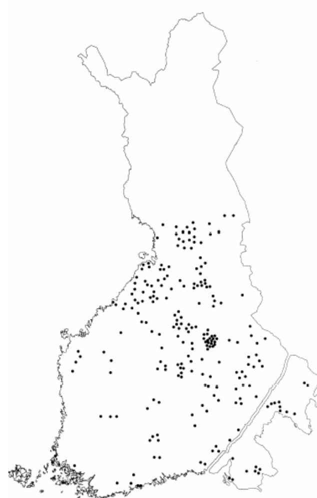


Figure 6. The birthplaces of the great-grandparents of 31 CLD families in Finland. The figure is adapted from Järvelä et al. (1998).

2.3.1 Clinical features

CLD is characterized by a pure watery diarrhea that is due to osmosis developed by unhydrolysed lactose. Feces are acidic (pH 4.5-5.5) and contain large quantities of lactose as a result of undeveloped bacterial flora. Later, at the age of 1.7 years or older lactose is absent, probably fermented by colonic bacterial flora creating flatulence and abdominal pains. The severe diarrhea followed by dehydration,

metabolic acidosis, and weight loss is usually diagnosed during the first weeks or months of life (Savilahti et al. 1983). Some patients have hypercalcemia and medullar nephrocalcinosis (Saarela et al. 1995). Serum cholesterol is reduced but the levels of triglycerides are normal. Regardless of the symptoms, CLD infants are lively and they have a good appetite (Savilahti et al. 1983). Lactase activity determined in duodenal biopsy specimen is very low (0-10 U/g protein). The morphology of duodenum and sucrase, maltase and isomaltase activities are observed to be normal. Normal psychomotor development and growth is achieved and the symptoms disappear when the patients are put on a lactose-free diet (Savilahti et al. 1983).

2.3.2 Assignment of the locus to 2q21

As lactase is responsible for the hydrolysis of lactose into galactose and glucose in the intestinal lumen it was an obvious candidate for CLD. *LCT* was localized to chromosome 2q and subsequently 2q21 by Kruse et al. (1988) and Harvey et al. (1993), respectively (Figure 7). Using this information Järvelä and colleagues (1998) found linkage for CLD on 2q21 in the close proximity of the *LCT* gene in 19 Finnish families. However, extended haplotype analysis by seven polymorphic microsatellite markers seemed to refine the CLD locus in the 350 kb area between D2S314 and D2S2385 from two centiMorgans (cM) telomeric from *LCT* (Figure 7). These microsatellites gave the highest LD in the disease alleles. This finding supported the hypothesis of one major mutation in most CLD patients in Finland. Based on strong LD spanning an area of ~8 cM at the CLD locus, the mutation was estimated to have been enriched by approximately 30 generations in the Finnish population (Järvelä et al. 1998). Sequence analysis of *LCT* and its promoter region failed to reveal disease-causing mutations in a Finnish CLD patient (Poggi and Sebastio 1991). This finding and the haplotype analyses of Finnish CLD patients encouraged the authors to suggest that there is a novel gene underlying CLD (Järvelä et al. 1998).

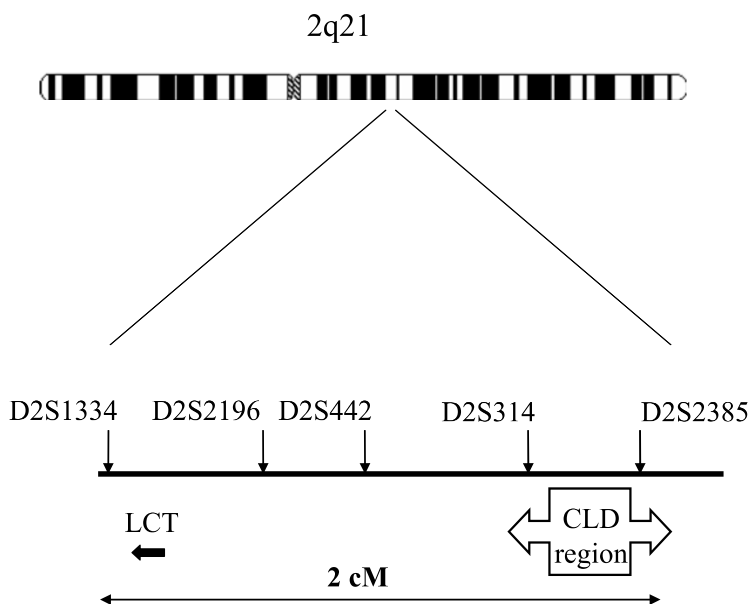


Figure 7. Physical map of the CLD region. According Järvelä et al. (1998) the critical CLD region located 2 cM telomeric from the *LCT* gene.

2.4 Adult-type hypolactasia

Adult-type hypolactasia (MIM 223100) (lactase non-persistence, lactose intolerance) is an autosomal recessive gastrointestinal condition that is the result of a decline in the activity of lactase in the intestinal lumen after weaning. Downregulation of lactase is considered as a normal phenomenon among mammals, and symptoms are remarkably milder than experienced in CLD. Milk intolerance was described in 1901 when carbohydrate ingestion was noted to be linked with pathogenesis of diarrhea (Montgomery et al. 1991). Röhmann and Nagano (1903) demonstrated the pathophysiologic mechanism of this condition in dogs as early as 1903 when they observed unhydrolysed lactose molecules in the intestinal lumen, reviewed in Sahi (1994a). The lactase activity was detected in the duodenum but the activity was highest in the jejunum and decreased towards the ileum. Absorption was observed in

the jejunum (Borgström et al. 1957). In 1963, Auricchio et al. (1963) and Dahlqvist et al. (1963) reported a decrease in lactase activity from birth in humans. Interestingly, some humans have the ability to maintain lactase activity and digest lactose throughout their lives (lactase persistence). The reasons for these phenomena have been speculated on for a quite some time. It was noted that lactase activity could not be induced by intake of lactose (Keusch et al. 1969b). Genetic factors were observed to play a role when evidence for the autosomal recessive inheritance was published in 1973 (Sahi et al. 1973). These fascinating characteristics have inspired researchers to study the molecular background of the lactase non-persistence and persistence phenotypes.

2.4.1 Diagnosis

Diagnosis of adult-type hypolactasia is commonly assessed by the lactose tolerance test (LTT). The method is indirect and is based on a serial of measurements of glucose in blood after oral lactose ingestion (50 g). A small rise in blood glucose concentration (<1.1 mmol/l) after a 20, 40, 60 and 90 minutes of lactose load indicate hypolactasia. A rise in blood glucose of more than 1.7 mmol/l indicates lactase persistence. In addition, clinical symptoms, such as possible stomach pains, distension, cramps, flatulence, nausea and diarrhea are registered (Sahi and Launiala 1978; Arola 1994). Lactose tolerance test with ethanol (LTTE) was a successful step towards a more specific test. Galactose in blood is measured instead of glucose and ethanol is used to inhibit the conversion of galactose to glucose in the liver (Jussila 1969; Isokoski et al. 1972; Arola 1994). One measurement at 40 min after lactose and ethanol ingestion is sufficient for a diagnosis. Blood galactose concentrations less than 0.3 mmol/l indicate hypolactasia (Isokoski et al. 1972). In addition, there are some applications of LTTE, which rely on urinary galactose determinations, reviewed in detail in Arola (1994). Tolerance tests for glucose and galactose are needed for exclusion of secondary malabsorption (Jussila 1969). Diagnosis by the

breath hydrogen test (BHT) after lactose ingestion is based on the definition of hydrogen from exhaled air. Hydrogen concentration is measured by gas chromatography from a set of samples taken for 2 to 6 hours. Usually hydrogen concentration >20 ppm indicates hypolactasia (Metz et al. 1975; Metz et al. 1976; Arola 1994). However, a definitive diagnosis of adult-type hypolactasia can be made by measuring lactase together with sucrase and maltase activities directly from intestinal biopsy specimen and exploring the histology of the mucosa (Dahlqvist 1984). Lactase activity less than 10 U/g protein and a ratio of lactase to sucrase activities less than 0.30 indicate hypolactasia (Jussila 1969; Dahlqvist 1984). Histological evaluations are important to study possible secondary reasons for a low lactase activity. Infections and inflammations have been found to decrease activities of several intestinal enzymes (Phillips et al. 1988). The comparison of indirect LTT, different LTTEs and BHT with direct mucosal disaccharidases shows that their sensitivities and specificities vary between 69 to 100% (Isokoski et al. 1972).

2.4.2 Onset and prevalence

In a number of populations lactase activity starts to decline a few years after weaning. The age at downregulation varies between populations. For example in Thais downregulation takes place between 1-2 years of age (Keusch et al. 1969a). In African children, the first signs of decline of lactase activity was detected at 3 years of age but in Finnish children somewhat later at 5-12 years of age (Rasinperä et al. 2004). A British study of different ethnic groups demonstrated that downregulation of *LCT* expression was detected from the second year of life, although the extent and onset was not constant (Wang et al. 1998b). The reasons for these timing variations are unknown.

The prevalence of adult-type hypolactasia varies between populations. The prevalence of adult-type hypolactasia is highest in Asia, for example 90-100% of

Thais, Chinese, and Japanese suffer from a decline of lactase activity (Flatz and Saengudom 1969). A total of 81-91% of the blacks of Africa cannot digest lactose (Cook and Kajubi 1966; Olatunbosun and Kwaku Adadevoh 1971). Lactase non-persistence is a frequent phenotype among native population of Australia and America reviewed in Swallow and Hollox (2000). In contrast, a low prevalence of hypolactasia 1-9.6% have been observed in Sweden and Denmark (Gudmand-Höyer et al. 1969; Dahlqvist and Lindquist 1971; Nilsson TK personal communication). Also, some nomadic populations in Africa and Arabia such as the Beja, Beduin, Fulbe and Tuareg can tolerate lactose efficiently, only 0-24% of them develop hypolactasia, reviewed in Swallow and Hollox (2000). Among the Finns the prevalence is about 17-18% while the prevalence in Finnish Swedish-speaking population is only 8% (Jussila et al. 1970; Sahi 1974; Enattah et al. 2002).

2.4.3 Genetics

2.4.3.1 Evolutionary favour of lactase persistence

Lactase non-persistence is considered to be the ancestral phenotype, lactase persistence has been regarded to be evolutionary advantageous when milk from domestic cows became available as a source of nutrition (Hollox 2005). It has been rationalized that this gene-culture coevolution created selective pressure for individuals who could efficiently use milk for nutrition in adulthood. This has been supported by observations as dairy farming and lactase persistence coincide, by the epidemiologic data as there are a wide distribution of the prevalence in lactase persistence between populations and by the fact that lactase persistence is genetically determined (Simoons 1969; McCracken 1970; Simoons 1970; McCracken 1971; Sahi et al. 1973; Sahi 1994a; Enattah et al. 2002). The gene-culture coevolution hypothesis was strongly supported when a high diversity in cattle milk protein genes and lactase persistence was demonstrated to coincide in

Europe (Beja-Pereira et al. 2003). Furthermore, convincing lines of evidence for the selective pressure of lactase persistence were obtained when genetic data nearby *LCT* was evaluated in northern European or derived populations. The lactase persistence locus was observed to contain an exceptionally long haplotype, LD was detected up to 1 Mb (Hollox et al. 2001; Enattah et al. 2002; Poulter et al. 2003; Bersaglieri et al. 2004) which was demonstrated to be longer and more common (77% of northern Europeans) than expected only by chance (Bersaglieri et al. 2004). The strong selection was estimated to have been taken place during past the 10000 years, 400 generations coinciding with a dairy culture (Bersaglieri et al. 2004; Coelho et al. 2005; Myles et al. 2005; Enattah et al. unpublished). The selection power was calculated to be 1.4-15% which is in agreement with previous estimations of 1-7% (Cavalli-Sforza 1973; Heston and Gottesman 1973; Flatz and Rotthauwe 1977; Aoki 1986; Flatz 1987). These findings indicate that lactase persistence has undergone the strongest positive selection seen in the human genome (Bersaglieri et al. 2004). Indeed, genetic, cultural and epidemiologic signs indicate that the ability to use milk efficiently for nutrition improved the survival opportunities of early farmers.

2.4.3.2 Mechanism of downregulation

Mutation analysis of *LCT* and its one kb sized promoter area have not shown the sequence variation associated with adult-type hypolactasia (Boll et al. 1991; Lloyd et al. 1992). In addition, a number of single nucleotide polymorphisms (SNPs) have been identified in one Mb fragment of LD at *LCT* but none of them could explain the lactase persistence phenotype (Harvey et al. 1995; Harvey et al. 1998; Hollox et al. 2001; Poulter et al. 2003). Many studies have been conducted at the cellular level to explain the phenotype differences and as a result several factors have been reported to influence the decline of lactase after childhood. At first, posttranscriptional regulation was suggested when *LCT* mRNA levels could not be

seen to correlate with lactase activity (Sebastio et al. 1989). Both slow processing of the lactase protein or/and reduction of pro-LCT synthesis were observed in metabolic labelling studies in lactase non-persistent individuals (Sterchi et al. 1990; Witte et al. 1990; Lloyd et al. 1992). Biosynthesis of pro-LCT has been detected to correlate with *LCT* mRNA levels but not lactase activity. A comparison of *LCT* mRNA levels and lactase activity/*LCT* mRNA level ratios indicated a heterogeneous pattern of regulation in both hypolactasic and lactase persistent individuals (Rossi et al. 1997). Even, mosaic regulation of lactase was observed from individuals with adult-type hypolactasia (Maiuri et al. 1994). However in the majority of cases, the *LCT* mRNA level have been shown to correlate with lactase activity or the ratio of lactase to sucrase (L/S) activities indicating that the decline of lactase activity is regulated at the transcriptional level (Escher et al. 1992; Lloyd et al. 1992; Fajardo et al. 1994). Analogous results have been observed in studies on animals (Krasinski et al. 1994; Lacey et al. 1994). Later, the lactase persistence/non-persistence was demonstrated to be controlled by a cis-acting element (Wang et al. 1995). In that study, *LCT* steady state mRNA levels were studied utilizing SNPs in the coding region of *LCT*. Certain types of lactase persistent were detected to have asymmetric allelic mRNA expression suggesting that expression of the *LCT* alleles could be independently regulated. Authors concluded that a developmentally regulated trans-acting DNA-binding protein could bind to only one kind of lactase allele and influence transcription and/or mRNA stability (Wang et al. 1995; Wang et al. 1998b).

2.4.3.3 Discovery of the responsible variant

Enattah and colleagues (2002) used the candidate gene approach and restricted the locus of adult-type hypolactasia to a 47 kb area at the 5'-end of the *LCT* gene on 2q21 using LD and haplotype analysis of nine extended Finnish families. Diagnoses for adult-type hypolactasia were assessed by LTTE. Sequence analysis of the 47 kb region exposed two SNPs C/T_{-13910} (rs4988235) and G/A_{-22018} (rs182549), 14 kb and

22 kb upstream from the initiation codon of *LCT*, respectively. The SNPs C/T$_{-13910}$ and G/A$_{-22018}$ are located in introns 13 and 9 of the minichromosome maintenance deficient 6 (*MCM6*) gene, respectively (Figure 8). These SNPs cosegregated completely with the adult-type hypolactasia trait in Finnish families. All hypolactasic individuals showed homozygosity for C$_{-13910}$ and G$_{-22018}$. Furthermore, in an independent sample set of 236 individuals, with biochemically verified disaccharidase activities from intestinal biopsy specimens from four different populations, the C/T$_{-13910}$ SNP was completely found to be associated with the trait and the SNP G/A$_{-22018}$ associated in 229 of 236 cases (Enattah et al. 2002). The frequency of the C/C$_{-13910}$ in a sample set of 1047 individuals was in agreement with the reported prevalence of adult-type hypolactasia in Finnish, French, African American and South Korean populations and North American Caucasians (Sahi 1974; Simoons 1978; Cuddenec et al. 1982). As early as this time, the lactase persistence allele, T$_{-13910}$, was anticipated to be very old as it was found in distally related populations (Enattah et al. 2002).

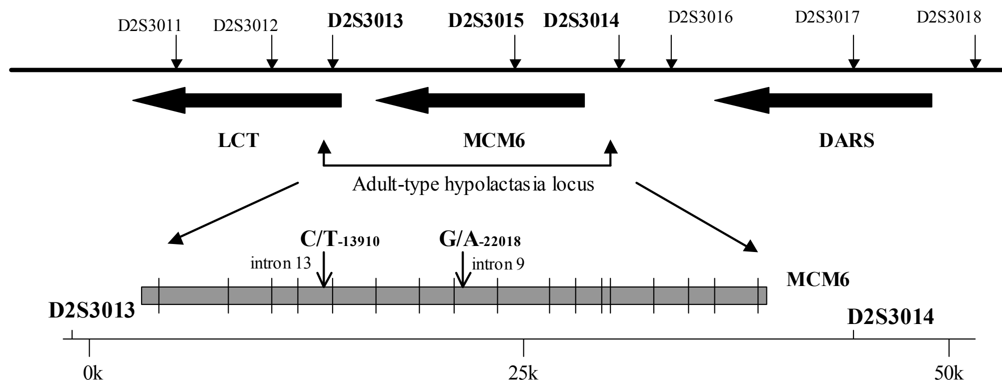


Figure 8. The physical map of the adult-type hypolactasia locus. The adult-type hypolactasia locus is located between markers D2S3013 and D2S3014. The horizontal arrows indicate the positions of lactase (*LCT*), minichromosome maintenance deficient 6 (*MCM6*) and aspartyl-tRNA synthetase (*DARS*). SNPs C/T$_{-13910}$ (rs4988235) and G/A$_{-22018}$ (rs182549) are located in intron 13 and 9 of the *MCM6* gene, 14 and 22 kb from *LCT*, respectively. Modified from Enattah et al (2002).

2.4.3.3.1 Functional evidence

Both the SNPs, C/T_{-13910} and G/A_{-22018}, were found in introns of *MCM6*, which are located in close proximity of, only 2.5 kb upstream, *LCT*. MCM6 is a mammalian homologue of mis5 of yeast, it functions as a cell cycle factor (Takahashi et al. 1994) and its mammalian version was initially identified in intestinal crypt cells of the rat (Sykes and Weiser 1995). *MCM6* is expressed in various human tissues including intestine during development. However, intestinal expressions of *MCM6* and *LCT* were not observed to match with lactase persistence or non-persistence individuals suggesting that these genes are independently regulated (Harvey et al. 1996). Thus, it seems that the influence of the *MCM6* gene on adult-type hypolactasia is only structural. The next evident step was to define the significance of C/T_{-13910} and G/A_{-22018} on the regulation of lactase activity. The answer for the dilemma was sought in the present study (III, see discussion) but later also by others using human (Troelsen et al. 2003b) and rat (Olds and Sibley 2003) LCT promoter-reporter gene construct analyses in Caco-2 cells. Both C_{-13910} and T_{-13910} variants were observed to enhance LCT promoter activity. The region containing T_{-13910} was demonstrated to increase 25%-75% LCT promoter activity compared to the results with the region cloned with C_{-13910} in undifferentiated Caco-2 (Olds and Sibley 2003; Troelsen et al. 2003b). Interestingly, the enhancer activity of C_{-13910} and T_{-13910} was detected to be several times higher in differentiated Caco-2 cells. The differentiated Caco-2 cells are commonly considered as a better model as they endogenously express *LCT*. Also, the difference of enhancer activities between C_{-13910} and T_{-13910} was found to be clearer, the T_{-13910} variant had a 3-6-fold increase in transcription compared with the C_{-13910} variant. Furthermore, electrophoretic mobility shift assays (EMSAs) showed a strong interaction between the T_{-13910} variant and a nuclear factor in nuclear extracts of HeLa and differentiated Caco-2 cells. On the contrary, the C_{-13910} variant had a weak interaction suggesting that the observed difference of LCT promoter activities are due to the binding capacity of C_{-13910} and T_{-13910} of a nuclear factor (Troelsen et al. 2003b). Analogous analyses of

the G/A_{-22018} variant with human and rat LCT promoters did not reveal any significant functional role for phenotype differences of adult-type hypolactasia (Olds and Sibley 2003; Troelsen et al. 2003b). The G/A_{-22018} variants were detected to possess a modest silencer effect on the C/T_{-13910} enhancer activity but there were no differences where one of the variants G_{22018} or A_{-22018} were present (Troelsen et al. 2003b). These results suggest that only C/T_{-13910} has a functional significance for enhancing *LCT in vitro* (Olds and Sibley 2003; Troelsen et al. 2003b). Although, G/A_{-22018} was observed to closely associate with the adult-type hypolactasia trait (Enattah et al. 2002) it probably is in LD locating only 8 kb from C/T_{-13910}.

The hunt for the nuclear factor, which binds to the C/T_{-13910} enhancer, was started from nuclear extracts of HeLa and CaCo-2 cells. DNA affinity purification analyses using the DNA region containing the T_{-13910} variant demonstrated co-purification of Octamer-binding transcription factor-1 (Oct-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Super shift assays showed that Oct-1 binds directly to the T_{-13910} variant. Furthermore, the binding was detected to be stronger to the T_{-13910} variant than the C_{-13910} variant (Lewinsky et al. 2005). Earlier, GAPDH has been shown to directly interact with Oct-1 (Zheng et al. 2003) which could explain its observed co-purification with Oct-1 (Lewinsky et al. 2005). In addition, DNase footprint and supershift analyses identified binding motifs for Cdx-2, GATA-6, Fox and HNF-4α nearby C/T_{-13910} (Figure 9). Mutation analyses of the Oct-1 binding site of T_{-13910} enhancer led a 5-fold decrease of expression using LCT promoter-reporter gene construct in Caco-2 cells. Also, mutation analyses of GATA, Fox and HNF-4α sites remarkably diminished the T_{-13910} enhancer activity suggesting they are important factors belonging to the enhancer structure; in contrast mutations in the Cdx-2 site had no significant effect on enhancer activity. Co-transfection analyses of Oct-1, GATA-6, HNF-4α, Cdx-2 and HNF-1α and HNF-1β with the T_{-13910} enhancer uncovered the fact that Oct-1 triggers reporter gene expression (133-fold) only with HNF-1α. The HNF-1α motif has not been found within the C/T_{-13910}

enhancer but it probably acts via a proximal promoter, which has a fine-characterized HNF-1α binding site. Co-transfection of Oct-1 and HNF-1α using the C_{-13910} enhancer led to a 112-fold increase in enhancer activity. Over-expression of Oct-1 could not alone initiate the T_{-13910} enhancer suggesting Oct-1 is not the limiting factor in Caco-2 cells. Over-expression of HNF-1α, GATA-6, HNF-4α and Cdx-2 was observed to induce the C/T_{-13910} enhancer activity but only HNF-1α was observed to maintain the difference in activity between the T_{-13910} and C_{-13910} enhancers. In addition, the C/T_{-13910} enhancer had no influence on MCM6 promoter activity. The results suggest that the C/T_{-13910} variant locates in the middle of a sophisticated enhancer structure consisting of at least Oct-1, GATA, Fox and HNF-4α binding sites (Figure 9). Oct-1 with C/T_{-13910} directs specifically the LCT promoter activity and enhances reporter gene expression with the T_{-13910} variant significantly over the C_{-13910} variant. These *in vitro* observations offer a convincing model for T_{-13910}-induced lactase persistence in humans (Lewinsky et al. 2005).

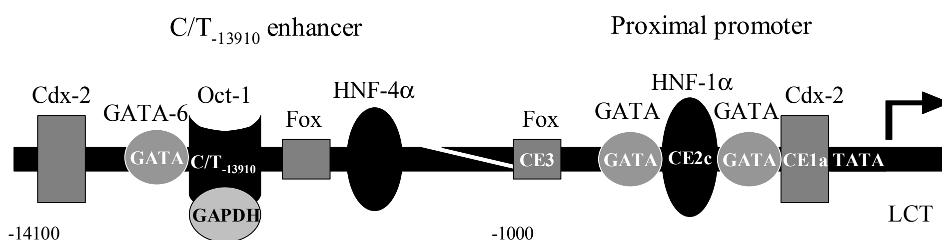


Figure 9. Structure of the C/T_{-13910} enhancer and the proximal promoter. Analysis of region of the C/T_{-13910} enhancer revealed the binding sites for Cdx-2, GATA-6, Oct-1, Fox and HNF-4α from 14045-13825 bp upstream of the *LCT* gene. Oct-1 interacts directly with the C/T_{-13910} site and has a stronger affinity to the T_{-13910} than the C_{-13910} variant. Oct-1 was observed to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that possibly functions as a co-activator for Oct-1. Mutation analyses of the binding sites demonstrated that the GATA-6, Fox and HNF-4α sites belong to the enhancer structure together with the Oct-1 site eliminating the enhancer activity. Disrupt of the Cdx-2 binding site had no effect on the enhancer activity. In expression studies of the C/T_{-13910} enhancer and the proximal LCT promoter construct, Oct-1 was observed to enhance reporter gene expression only when co-expressed with HNF-1α. This is assumed to occur through interactions with Oct-1 and HNF-1α that is bound to the CE2c site at the proximal promoter (Lewinsky et al. 2005).

2.4.3.4 T_{-13910}, the only variant ruling lactase persistence for humanity?

Originally, the T_{-13910} variant was cloned in the Finnish population, which is a population that has been proven to be invaluable for tracing mutations underlying rare monogenic diseases (Enattah et al. 2002). Interestingly, the variant has also been found in other European Caucasians or their descendants. The prevalence of the T_{-13910} allele has been observed to be in agreement with the epidemiological observations of lactase persistence in Austria, (Obermayer-Pietsch et al. 2004; Bodlaj et al. 2006) Germany, (Büning et al. 2003) France, US, (Enattah et al. 2002) UK, (Rasinperä et al. 2005) Ireland (Mulcare et al. 2004) Poland and Sweden (Study IV). C/T_{-13910} has also been shown to match, with few exceptions, with sets of individuals whose ability to hydrolyse lactose was determined by duodenal enzyme assays in the UK (Poulter et al. 2003) and by LTT in Sweden (Nilsson and Johansson 2004; Ridefelt and Håkansson 2005). In Austria, BHT diagnosed lactase status correlated with the C/C_{-13910} genotype, however the test indicated lactase deficiency in 14% of individuals with C/T_{-13910} and T/T_{-13910} (Hogenauer et al. 2005). The results are acceptable if it is taken into account that the accuracy of BHT is not very high (Arola 1994). Outside of Europe the T_{-13910} allele was strongly represented in Pakistan and Algeria, lower frequencies were observed in Middle Eastern populations (Bersaglieri et al. 2004). In Pakistan, five out of eight population groups had the T_{-13910} allele frequency $\geq$0.30 (Bersaglieri et al. 2004) that is in scale with the phenotyped data (0.20-0.33) in this region (Abbas and Ahmad 1983; Ahmad and Flatz 1984). There is no published data of lactase persistence available for Algerians. The T_{-13910} allele was suggested to have been spread between populations along dairy farming thus, increasing traces of T_{-13910} is seen in neighbouring European populations (Bersaglieri et al. 2004). There are certain nomadic populations with known pastoralism and high lactase persistence in Africa and the Arabian Peninsula. To shed some light on lactase persistence in Africans, different populations were analysed for the T_{-13910} variant. Statistically, T_{-13910} could

not match with five out of seven population groups with the published lactase persistence incidences. Four of those five populations came from Sudan one came from Senegal. On the contrary, in two population groups from Cameroon T_{-13910} associated with the epidemiological observations. Based on these results, these five groups may have a divergent genetic background for lactase persistence. A variant that could explain lactase persistence in these groups was not identified (Mulcare et al. 2004). The study has its limitations including the small number of analysed subjects and carefully characterized phenotypes are required to confirm the results. As there may be other variants apart from the T_{-13910} one influencing lactase persistence, at least in Africans, it will be interesting to see whether any new variant found shares the same enhancer element or if its mechanism of up-regulation is more complex. Also, it is to be determined what variant nomadic populations of Arabia possess to allow lactase expression in adulthood. Be that as it may, the case is not closed yet. There is still work to be done to uncover causes or to confirm the present one for lactase persistence globally. Thus far, the T_{-13910} variant is the only one that has been published to be causative for the lactase persistence phenotype.

There have been discussions of the diagnostic value of the C/T_{-13910} variant for adult-type hypolactasia (Swallow 2006). Based on studies in children (Rasinperä et al. 2004) and the population and functional studies reviewed here, C/T_{-13910} can be regarded as a suitable first-stage screening test for adult-type hypolactasia in adults of European ancestry.

2.5 Lactase persistence, non-persistence and dairy consumption risk factors for other diseases?

Milk contains a large quantity of calcium, lactose and many other components such as vitamin D and various vitamins of B. Milk is a versatile nutrient, and its consumption and effects on health have been studied with a passion. Obviously high

lactase activity facilitates the use of milk as nutrition and this has been noted as an increasing milk consumption in western countries (Obermayer-Pietsch et al. 2004; Rasinperä et al. 2004; Rasinperä et al. 2006). Hence, lactase persistence or non-persistence has been suggested to influence indirectly the pathogenesis of certain diseases. Perhaps one of the most studied is the link between the milk consumption and the development of osteoporosis. Calcium is the main mineral involved in bone formation and it is needed throughout life. The recommended daily intake of calcium is about 900 mg/day for adults, 1200 mg/day for adolescents and the elderly (National Nutrition Council 1999). As milk is an excellent source of bioavailable calcium, it has a positive effect on calcium balance and therefore on bone formation. Especially, the consumption of dairy products in childhood and adolescence is seen as an increasing bone density in adulthood and an ability to protect women against osteoporosis (Gueguen and Pointillart 2000). In addition, the C/C_{-13910} genotype defining lactase non-persistence associated with bone fractures in elderly people in Austria and Finland (Obermayer-Pietsch et al. 2004; Enattah et al. 2005b) but in young Finnish men the genotype had no effect (Enattah et al. 2004a). With this background lactase persistence could help maintain the milk drinking habit from childhood to adolescence and hence decrease the risk of osteoporosis. However, studies focussed on this issue are controversial and there is not consensus among researchers. Even, a case control study using genetically defined lactase status by the C/T_{-13910} variant could not find any link between lactase non-persistence and osteoporosis in Finnish postmenopausal women (Enattah et al. 2005a). Lactose has also been suggested to enhance calcium absorption in the intestine but studies on subjects with lactase persistence and non-persistence have shown no real proof of this. In general, it has been rationalized that diminished calcium availability in non-persistence subjects might explain the more efficient calcium absorption, reviewed in Gueguen and Pointillart (2000), Prentice (2004). Osteoporosis is a complex disease, which is caused by genetic and environmental factors. It seems that dietary calcium for its part during the peak of bone formation reduces bone fracture risk and

osteoporosis in adulthood. Yet, the effect of lactase non-persistence is more arguable (Gueguen and Pointillart 2000; Prentice 2004). The composition of Finnish 1.5% milk is represented in Table 2 (Fineli, National Public Health Institute, Finland).

Table 2. Milk (1.5%) content

Milk component	Quantity	Milk component	Quantity
Energy	191 kJ	Iodine	16.0 µg
Fat	1.5 g	Selenium	2.8 µg
Protein	3.0 g	Vitamine A	12.9 µg
Lactose	4.8 g	Vitamine D	0.5 µg
Cholesterol	6.4 mg	Vitamine E	<0.1 mg
Sodium	41.0 mg	Vitamine K	0.33 µg
NaCl	104.5 mg	Vitamine C	1.1 mg
Potassium	150.0 mg	Folate	4.2 µg
Magnesium	11.0 mg	Niasine	0.8 mg
Calcium	120.0 mg	Vitamine B2	0.19 mg
Phosphorus	90.0 mg	Vitamine B1	0.04 mg
Iron	< 0.1 mg	Vitamine B12	0.4 µg
Zinc	0.4 mg	Carotenoid	8.7 µg

Milk consumption has been proposed to have a modest protective effect against colorectal cancer (World Cancer Research Fund 1997). Primarily, the protective effect has been suggested to be mediated by calcium and vitamin D. However, only large cohort studies have been able to confirm the relation, most case control studies have failed to find supportive evidences for the hypothesis (Norat and Riboli 2003). Lactase persistence variant, T_{-13910}, was found to have a modest protective effect against colorectal cancer in the Finnish population. However, there was no significant risk effect observed in the British or Spanish populations (Rasinperä et al. 2005). This might be related to the fact that Finnish milk consumption is the highest in the world (The World Dairy Situation 2003). The findings require more detailed studies concerning the possible mechanism between components of milk and colorectal carcinogenesis. Therefore, another association study would not seem to be the most reasonable alternative.

Lactase non-persistence has been suggested to associate also with Crohn's disease (Mishkin et al. 1997; von Tirpitz et al. 2002) and lactase persistence with type I and II diabetes (Meloni et al. 2001). Association analysis of C/T_{-13910} with patients with Crohn's disease (Büning et al. 2003) and type I and II diabetes (Enattah et al. 2004b) did not support those earlier findings. These and other's findings (Park et al. 1990) suggest that the hypotheses might be closer to a myth than a real cause and effect relation.

2.5.1 Ovarian carcinoma

Ovarian cancer is the sixth most common cause of cancer among women but it is one of the deathliest disease of the female genital tract in the world (Parkin et al. 2001; Schulz et al. 2004). Ovarian cancer occurrence rates are observed to be highest in Europe and North America and lowest in Asia (World Cancer Research Fund 1997). Epithelial ovarian cancer (ovarian carcinoma) consists of 90% of ovarian cancers; germ cell and stromal cell ovarian cancers are responsible for 10% of cases. Ovarian carcinoma can be divided into four major histological subtypes: serous, endometrioid, mucinous and clear cells tumours of which the serous subtype is the most common one consisting of about 40% of ovarian carcinoma tumours. Papillary serous, Brenner cell, undifferentiated adenocarcinomas and sarcomas represent a minority of the histological subtypes. The majority of ovarian cancer patients are postmenopausal women in their late 50's, reviewed by Schulz et al. (2004). About 90% of the cases of all types of ovarian cancers are sporadic and hereditary factors are proposed to describe 5-10% of cases (Runnebaum and Stickeler 2001; Schulz et al. 2004). The aetiology of ovarian carcinoma is poorly known but both genetic and environmental factors are involved. For example, in sporadic ovarian carcinomas inactivation of the *p53* tumour suppressor gene has been observed (Wen et al. 1999; Christie and Oehler 2006). The *BRCA1* and *BRCA2* genes are occasionally mutated in hereditary ovarian carcinomas (Menkiszak et al.

2003; Christie and Oehler 2006). Numerous "incessant" ovulation and hormonal factors have also been hypothesized to be involved in its pathogenesis (Risch 1998). Thus, factors that reduce ovulation cycles such as multiparity, lactation, tubal ligation and hysterectomy or use of oral contraceptives are associated with a decreased risk of ovarian carcinoma (Whittemore et al. 1992; Hankinson et al. 1993). Also, nutritional features have been suggested as contributory factors (Haenszel and Kurihara 1968; Schulz et al. 2004).

2.5.1.1 Dairy consumption and ovarian carcinoma risk

Dairy products have been hypothesized to play a role in the tumour genesis of ovarian carcinoma. Milk, as known, has a high content of lactose and it is further metabolized by lactase to glucose and galactose. Galactose is converted to glucose in the liver by the Leloir pathway (Xu et al. 1989; Reichardt and Woo 1991). The hypothesis was born in the 1980's when animal studies showed that high quantities of dietary galactose is poisonous to oocytes (Chen et al. 1981; Schwartz and Storozuk 1988). In addition, young women with galactosemia were observed to develop ovarian failure (Kaufman et al. 1981). Galactosemia is an autosomal recessive disorder with an almost total absence of galactose-1-phosphate uridyltransferase (GALT) activity leading metabolites of galactose to accumulate in the ovary and other tissues (Xu et al. 1989; Reichardt and Woo 1991). Hence, the premature ovarian failure was suggested to be due to the direct toxicity of galactose, its metabolites or elevated gonadotropin levels (Cramer and Welch 1983; Harlow et al. 1991; Leslie 2003). The hypothesis truly got off the ground when milk consumption and lactase persistence were observed to correlate with ovarian carcinoma incidence (Cramer 1989). Since, then a number of studies have been conducted to define the significance of milk or its components and/or lactase persistence/non-persistence to ovarian carcinogenesis. Once again the results are controversial. Lactase persistence and lactose consumption were shown to associate

with increased ovarian carcinoma risk in Sardinia, Italy (Meloni et al. 1999) and lactose intolerance was suggested to indicate a protective effect against ovarian carcinoma at an early age in eastern U.S. (Cramer et al. 2000) but in studies with subjects from Canada (Risch et al. 1994) and with Caucasian from the U.S. (Herrinton et al. 1995) no such association was found. Diagnosis of lactase phenotypes has been often obtained using LTTs and self-reports of clinical symptoms that naturally increase the distribution. In multiple studies, milk or lactose consumption was found to have a protective effect on ovarian carcinoma (Goodman et al. 2002a; Goodman et al. 2002b; Salazar-Martinez et al. 2002; Yen et al. 2003). Two cohort studies from the U.S. (Kushi et al. 1999; Fairfield et al. 2004) and one from Sweden (Larsson et al. 2004) found a correlation between lactose consumption and increased ovarian carcinoma risk, especially that of serous subtype (Fairfield et al. 2004; Larsson et al. 2004). In contrast, cohorts from the Netherlands (Mommers et al. 2006) and another from the U.S. (Kiani et al. 2006) did not show any association whatsoever. Several case control studies have failed to find a clear relation of milk or lactose consumption to ovarian tumour genesis (Mettlin and Piver 1990; Engle et al. 1991; Risch et al. 1994; Herrinton et al. 1995; Webb et al. 1998; Britton et al. 2000; Bertone et al. 2001; Pan et al. 2004; Parazzini et al. 2004). Meta-analyses showed that only cohort studies found a link between milk/dairy/lactose consumption and an increased risk for ovarian carcinogenesis, but not case control studies (Larsson et al. 2006), nor the studies combined (Qin et al. 2005; Larsson et al. 2006). However, a recent pooled analysis of 12 cohort studies showed no statistically significant association between lactose consumption and the risk of ovarian carcinoma (Genkinger et al. 2006). Dietary vitamin D has been shown to protect from the development of ovarian carcinoma (Bidoli et al. 2001; Salazar-Martinez et al. 2002). In addition, calcium consumption has been observed to decrease ovarian carcinoma risk (Bidoli et al. 2001; Goodman et al. 2002b). Calcium, dietary vitamin D and endogenous vitamin D formation has been shown to downregulate the synthesis of parathyroid hormone (PTH) (Takeuchi et al. 1995;

Malabanan et al. 1998). PTH is involved in dietary calcium absorption increasing 1,25 dihydroxyvitamin D synthesis. It has been suggested that PTH is involved in cancer stimulating IGF-1 formation or acting as a co-mitogen in preneoplastic lesion expressing PTH receptors (McCarty 2000). However, all studies have not supported the potential protective effect of vitamin D and/or calcium (Tzonou et al. 1993; Kushi et al. 1999; Goodman et al. 2002b; Salazar-Martinez et al. 2002; Pan et al. 2004). The results about the dairy consumption as a risk factor for ovarian carcinogenesis are contentious. Milk consists of a huge number of various components and there is no clear consensus of the effect of any of them. However, it must be taken into account and they are difficult analyse individually.

The influence of GALT activity as directly involving the galactose hypothesis has been studied in the development of ovarian carcinoma. Some studies have used the N314D polymorphism of GALT as a molecular marker as it has been shown to diminish the GALT activity to 74-43% from normal (Elsas et al. 1994; Langley et al. 1997). Decreased activity of GALT together with lactose consumption was detected to associate with ovarian carcinoma (Cramer et al. 1989). However, later studies could not find any significant difference in GALT activity (Herrinton et al. 1995; Webb et al. 1998; Cramer et al. 2000; Goodman et al. 2002a) or in the frequency of N314D (Goodman et al. 2002a) between ovarian carcinoma patients and controls. The frequency of N314D was found to be higher in women with serous ovarian carcinoma but not endometroid or clear cell subtypes (Morland et al. 1998). On the contrary, another study found that N314D associated with endometroid and clear cell ovarian carcinomas but not with the serous subtype (Cramer et al. 2000). In addition, the N314D polymorphism and lactose consumption associated with borderline ovarian carcinoma (Cozen et al. 2002).

2.6 Identification of disease genes

2.6.1 The Human Genome Project

The Human Genome Project (HGP) had the ambitious goal to determine the structure of the human DNA sequence. The HGP was initiated in various National Institutes of Health and the U.S. Department of Energy in the United States in 1990. In 1998 a private company, Celera Genomics, announced a three-year project aimed at revealing the human genome nucleotide sequence. In February 2001 they and the public International Human Genome Sequencing Consortium published a nearly complete sequence of the euchromatic portion of the human genome (Lander et al. 2001; Venter et al. 2001).

2.6.2 The Human Genome

The genetic map length of the human genome (unit of Morgan) between two loci defines the average number of genetic recombinations (crossing-over) occurring in a gamete during meiosis. The recombination frequency fluctuates with chromosomal regions and it is different for males and females. The genetic length of the whole genome is 4301 cM in females and 2849 cM in males (Collins et al. 1996). One centiMorgan is estimated to be roughly equal to a physical length of 1 Mb (Ott 1999).

The physical length of the euchromatic portion of the haploid human genome is ~2.91 billion bp. Sequence analysis showed circa 24500-26000 protein encoding transcripts and 6500-12000 additional genes obtained by gene prediction programs with weak similarities to other species. Exons cover only 1.1-1.5% of the genome, 24-33% is covered by introns, and 66-75% of the genome consists of intragenic DNA without any known function. The organization of the human genome primary

structure provides scientists with a tool to understand human biology (Lander et al. 2001; Venter et al. 2001).

2.6.3 Positional cloning

In positional cloning a disease gene is isolated based on its chromosomal location without any knowledge of its function or pathogenesis. A chromosomal location is defined by genetic mapping using family material adequate for linkage analyses. Locus can be further refined by LD and haplotype mapping (Collins 1992; Collins 1995). Today in the post genomic era, genes of interest are listed in databases, thus regional candidate genes can be carefully chosen and/or systematically screened for mutation.

2.6.4 Genetic mapping

Genetic mapping requires family material with a precisely diagnosed inherited disease. Only reliable diagnosis by experienced clinician(s) can lead to useful results using the genetic mapping approach. For linkage analysis two or more affected individuals are required. The collected family material is analysed with polymorphic microsatellite markers positioned at about 4-10 cM intervals throughout the genome. Until linkage is reached, the study can be further carried on by fine mapping with more frequent microsatellite maps to get as close as possible to the mutation responsible for the disease. Botstein et al. (1980) introduced the principles of genetic mapping with DNA markers in 1980 and Gusella et al. (1983) were the first to identify a human disease gene locus (Huntington disease) using this approach in 1983.

2.6.4.1 Genetic markers

There are a large number of sequence variations in the human genome between individuals. Most of them are located in the non-coding regions but such harmless alterations in the coding regions are not unusual. The segregation of these variations to offspring within families is the foundation for genetic mapping. The sequence variations are called polymorphisms when its most common allele has a population frequency of less than 95-99% (Botstein et al. 1980; Ott 1999). The usefulness of a marker for linkage analysis depends on the number of alleles and their gene frequencies (degrees of polymorphism). Short tandem repeats (STR) are highly polymorphic usually consisting of 10-50 copies of di-, tri-, tetra-, or pentanucleotide repeat elements. The most common repeat $(CA)_n$ is distributed approximately in 30 kb intervals in the human genome (Hearne et al. 1992). Today, thousands of microsatellite markers and several genetic maps are available in public databases (e.g. Marshfield Medical Genetic Foundation, Genome Database). High-resolution recombination maps have also been created and they are available as a supplementary file (Kong et al. 2002). The use of single nucleotide polymorphisms (SNPs) as biallelic markers have led to seriously new opportunities again not seen since the times of biallelic restriction fragment length polymorphism (RFLPs) detection. SNPs are observed about once in every kilobase, they are also found in the coding regions of the genes (cSNP). They are more stable than STRs but at the same time carry less information (Li and Sadler 1991; Wang et al. 1998a). SNPs can be easily and automatically identified using primer extension techniques and detected by scintillation, fluorometry or mass spectrometry based methods. They are suitable for genetic mapping and gene expression studies using cSNP as a marker (Syvänen et al. 1990; Syvänen et al. 1993; Lander 1996; Pastinen et al. 1996; Braun et al. 1997; Haff and Smirnov 1997; Pastinen et al. 1997; Ross et al. 1998; Syvänen 1998; Tang et al. 1999). Wang DG and colleagues (1998) published the first genetic SNP map in 1998.

2.6.4.2 Linkage analysis

The purpose of linkage analysis is to assign a disease locus its specific chromosomal region. It is based on comparing the inheritance of alleles of polymorphic markers with the disease phenotype. If a polymorphic marker is so close to a disease locus that they cannot be separated by recombination (or it is extremely rare) they are said to be genetically linked. The techniques of the statistical significance of linkage testing are based on maximum likelihood estimation and likelihood ratio testing. The most used approach is the logarithm of odds (LOD) score method. The likelihood (L) that two loci, e.g. a marker and a disease locus, are linked with a recombination fraction (θ) is compared the likelihood that they are not linked (θ=½). The LOD score Z(θ) is obtained when the ratio of these likelihoods are converted into $\log_{10}$,

$$Z(\theta) = \log_{10}\left[\frac{L(\theta)}{L(½)}\right].$$

A LOD score value ≥3, which means that two loci are 1000 times more likely to be linked than not linked, is considered to be significant evidence for linkage. On the other hand, a LOD score value ≤-2 is evidence against linkage (probably a 100 times likely not to be linked) (Morton 1955; Ott 1999). Linkage analyses are performed by comparing a disease locus with each marker locus as a two-point linkage analysis and analysis of numerous linked loci, multipoint analysis. Multipoint analysis uses the information of adjacent marker loci thus, the order of loci matters.

2.6.4.3 Linkage disequilibrium

Linkage disequilibrium is a phenomenon of non-random association of alleles at linked loci. The association can be statistically measured by the traditional chi-square (χ^2) test, the Fisher's exact test, (Armitage and Berry 1987) r^2, (Ardlie et al.

2002) and the likelihood based lambda (λ) test (Terwilliger 1995). The chi-square and a Fisher's exact tests evaluate independence of a 2x2 table. The chi-square test is adjusted for large sample sets.

$$\chi^2 = \sum_{i=1}^{R} \sum_{j=1}^{C} \frac{(O_{ij} - E_{ij})}{E_{ij}},$$

where O_{ij} is the observed frequency of the *i*th row and the *j*th column, E is the expected frequency of the *i*th row and the *j*th column, R is a number of rows and C is a number of columns (Armitage and Berry 1987). Lambda informs about the proportion of excess of a certain allele in disease chromosomes, relative to its population frequency.

$$\lambda_i = \alpha(1-\theta)^n ,$$

Where λ_i is proportion of excess of allele *i* in chromosomes carrying the disease allele, α_i is proportion of disease alleles originally in association with allele *i*, θ is recombination fraction, n is number of generation since introduction of the founder disease allele into the population (Terwilliger 1995).

LD is used for fine mapping of disease genes and it is powerful in situations where chromosomes are carrying a mutation originating from one common ancestor. An efficient LD mapping also requires a low number of the original settlers, rapid expansion of the population without major immigration like the Finns or an ancient small population which has been stable for a long time like the Saami, and a large enough number of patients (Edwards 1980; de la Chapelle 1993; de la Chapelle and Wright 1998; Terwilliger et al. 1998; Peltonen et al. 1999). The power of LD mapping was showed in a rare Finnish monogenic disease infantile-onset

spinocerebellar ataxia (IOSCA) where initial gene localization was obtained using four affected individuals from two consanguineous pedigrees (Nikali et al. 1995). LD mapping has also been successfully used in narrowing the disease locus in other rare Finnish diseases such as diastrophic dysplasia (DTD) (Hästbacka et al. 1992) or autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy (APECED) (Aaltonen et al. 1994). The length of LD can be utilized to estimate the age of mutations. Meiotic recombinations which may have occurred over time decrease the length of the observed LD generation after generation thus, indicating the older mutation (Varilo 1999). Figure 10 represents the principle used for LD mapping.

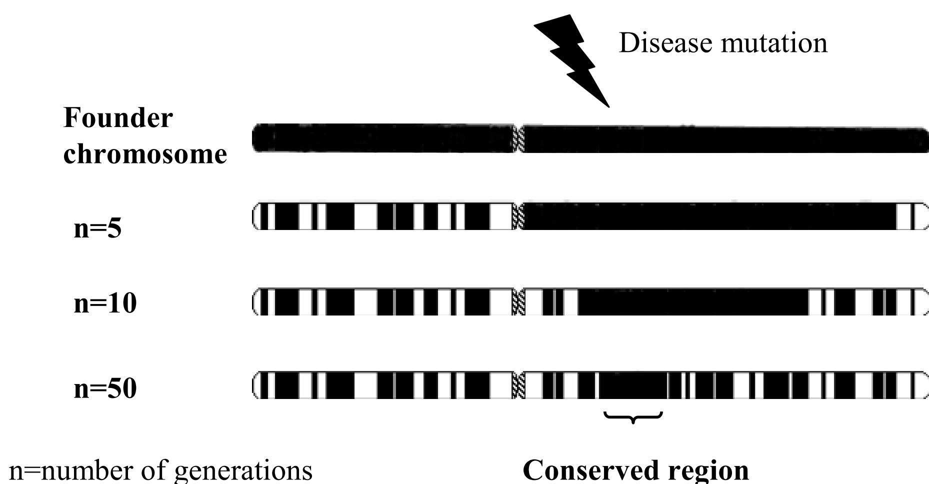


Figure 10. The basis of linkage disequilibrium (LD) mapping. The conserved region of a founder chromosome, exposed to disease mutation, shorten after generations due to recombinations in meiosis. In the course of time, only a short chromosomal segment is preserved from the founder chromosome. This event is detected as LD and can be used in a similar manner in haplotype analyses and for evaluating the age of mutation (de la Chapelle et al. 1994; Ott 1999; Varilo 1999; Norio 2000).

2.6.4.4 Haplotype analysis

Haplotype analysis, like LD mapping, is used for further restriction of disease locus in the isolated populations. Haplotype analysis is the identification of shared ancestral disease alleles of informative marker loci in a critical region. Recombinations events can dramatically reduce the shared region. Haplotype analysis extends beyond recombinations events by revealing old recombinations and thus further restricts the critical region needed to identify a disease gene, exemplified in many Finnish diseases such as progressive myoclonus epilepsy (EPM1) from about 7 cM to 0.30 cM (Lehesjoki et al. 1993).

2.6.4.5 Towards causative mutations

When a disease locus has been defined to a certain chromosomal region by genetic mapping, the virtually completed human genome project gives a possibility to use bioinformatics to locate ESTs, protein homologies, known genes or predicted genes. Also, genome projects of other organisms help to identify conserved gene structures and regulatory elements. Modern capillary techniques enable a fast mutation screening by cycle sequencing. The validation of found sequence variations can be performed by carrier screening, functional analyses *in vitro* and *in vivo*, and model organisms.

2.7 Gene expression studies

A fundamental part of disease gene characterization, as well as the basic gene studies, is to examine gene expression. Gene expression can be studied by a number of techniques for example, histochemically and immunohistochemically evaluations at the protein level. Cell specific gene expression can be detected using *in situ* hybridization at the mRNA level (Bleeker et al. 2000; Ropponen et al. 2001; van

Noorden 2002). Furthermore, more accurate sensitive expression detection techniques have been developed.

2.7.1 Techniques for mRNA detection

The monitoring of mRNAs has been found to be an important approach to studying gene function. Alwine et al. (1977) modified the southern blot technique (Southern 1975) and created the specific northern blot hybridization assay for the detection of mRNA. Northern blotting requires electrophoretic separation of total RNA or mRNA on an agarose gel, transferring to a nitrocellulose sheet, and hybridization with a ^{32}P-labeled DNA probe. The results are visualized by autoradiography.

Since Mullis et al's (1986) development of the PCR method, RT-PCR has been utilized efficiently to study the expression of genes. RT-PCR is a sensitive technique that enables the quantitative monitoring of changes of mRNA expression at the cellular level. Real-time PCR offers the opportunity to follow the accumulation of PCR products during amplification. It is based on specific hybridization of the fluorescent probes. Quantitation can be obtained using internal standard competitively during amplification and detection is based on decrease in fluorescein quenching by rhodamine after Taq DNA polymerase's exonuclease cleavage of dual-labelled probes during the exponential phase of amplification (Livak et al. 1995), see also (Holland et al. 1991). Alternatively, fluorescence can be monitored by the resonance energy transfer of fluorescein to Cy5 between adjacently hybridized probes (Wittwer et al. 1997).

A number of competitive endpoint RT-PCR based techniques have been developed. The quantitation is achieved by coamplification of *in vitro*-generated internal standard RNA with target mRNA. For example, in the early quantitation methods the PCR products of the internal standard and target mRNA had to differ in length (Wang et al. 1989) or the size difference were generated by restriction endonuclease

digestion (Becker-Andre and Hahlbrock 1989). The detection needed electrophoretic separation followed by northern blot hybridisation. The initial copy number of the target mRNA is estimated from the ratio to the internal standard. However, more sensitive methods of endpoint quantitation have been introduced. The versatile enzyme-linked immunosorbent (ELISA) PCR assay relies on the hybridization of a solid-support captured amplification product with a digoxigenin-labeled internal oligonucleotide probe. Detection is performed by optical reading after an anti-digoxigenin alkaline phosphatase coupled antibody treatment (Alard et al. 1993). Solid-phase minisequencing enables quantitation of highly homologous sequences that differ from each other only by one single nucleotide (Figure 11). It utilizes DNA polymerase to a single nucleotide primer extension. PCR is performed by a biotinylated primer which is immobilized to a streptavidine coated solid support and denaturated. A detection primer is designed to anneal adjacent to the single nucleotide polymorphism and minisequencing is performed separately with the expected ^{3}H-labeled dNTPs. The ratio of two incorporated labels as a measure of the two sequences is determined by a liquid scintillation counter (Syvänen et al. 1990; Syvänen et al. 1992a; Syvänen et al. 1992b). Solid-phase minisequencing has been used for quantitation of rare transcripts (Ikonen et al. 1991) and e.g. the detection method using fluorescent labels in multiplex analyses (Pastinen et al. 1996; Pastinen et al. 1997). Furthermore, solid-phase minisequencing was used for accurate determination of the relative mRNA levels of two closely related mRNAs (squamous cell carcinoma antigen; SCCA1 and 2). Quantitation was based on the ratio of the SCCA2 to SCCA1 genes in the same cell as internal standard for each other (Stenman et al. 1999), see also (Karttunen et al. 1996). Multiple genes or whole genomes can be simultaneously studied by the complementary DNA microarray technology. DNA microarray expression analysis involves reverse transcription (RT) of isolated mRNA with fluorescently labelled dNTPs. A mixture of equivalent amounts of both labelled cDNA sample are hybridized to the

microarray and the ratio of labels are measured by laser confocal fluorescent scanning (Schena et al. 1995; Lockhart et al. 1996; Brown and Botstein 1999).

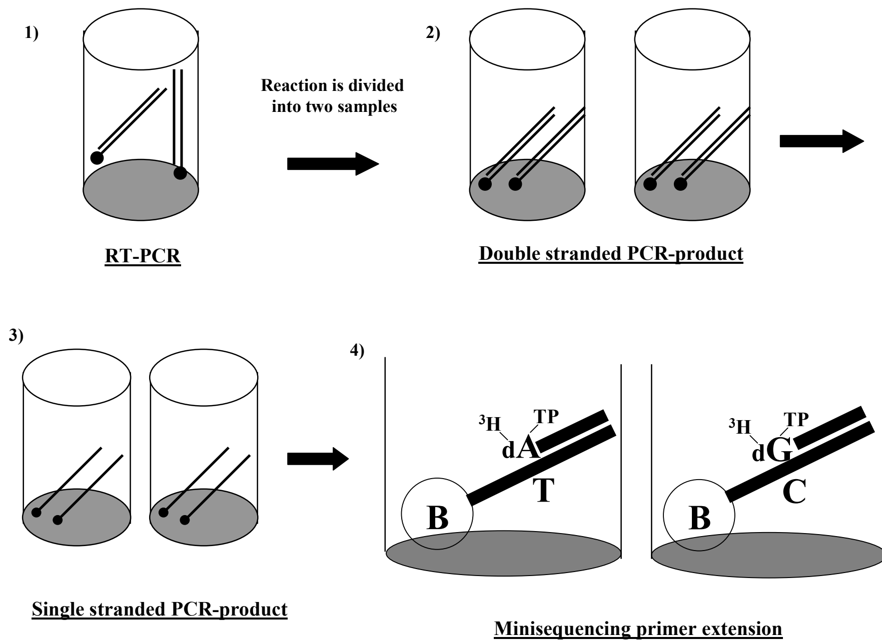


Figure 11. The principle of the solid-phase minisequencing. The initiation step is PCR or RT-PCR with a biotinylated primer. 2. Then dividing the reaction into two samples and immobilizing streptavidine-coated microplates. 3. Denaturation of the complementary strand. 4. A primer-guided tritium labelled nucleotide incorporation assay. As a result, the ratios of two nucleotides as a measure of two alleles are calculated (Syvänen et al. 1990; Syvänen et al. 1992a; Syvänen et al. 1992b). The Figure is modified from Study III.

3 AIMS OF THE STUDY

Aims of the present study were:

1. To identify causative mutations for congenital lactase deficiency (I).

2. To determine the functional consequences of the C/T$_{-13910}$ variant associated with adult-type hypolactasia both in children (II) and in adults (III).

4. To study a potential relationship between lactase persistence and ovarian carcinoma (IV).

4 MATERIALS AND METHODS

4.1 Study subjects

4.1.1 Finnish CLD families and controls (I)

Initially, family material consisted of 19 Finnish families, 27 patients and 64 healthy family members (Järvelä et al. 1998) at the end of the study a total number of 32 patients, 76 healthy family members from 24 families were included. The CLD diagnoses were based on clinical symptoms as described elsewhere (Savilahti et al. 1983). The carrier frequencies of the CLD mutations were determined from 556 anonymous blood donors obtained from the Finnish Red Cross Blood Transfusion Service.

4.1.2 *LCT* mRNA study in children (II)

Fifteen Finnish children and adolescents aged 10 months to 23 years from the Hospital for Children and Adolescents were included in the study.

4.1.3 *LCT* mRNA study in adults (III)

A total of 52 patients having unspecific abdominal complaints from Herttoniemi Hospital participated in the study. The diagnosis for adult-type hypolactasia was based on lactase and sucrase activities. An L/S ratio <0.30 indicated hypolactasia. Lactase, sucrase and maltase activities were determined from a duodenal biopsy specimen based on (Dahlqvist 1984). To exclude secondary lactase deficiencies all biopsies underwent histological examinations. Enzyme activities were determined at the Laboratory of the Hospital for Children and Adolescents.

4.1.4 Lactase persistence and ovarian carcinoma (IV)

The Finnish sample set consisted of 327 patients with ovarian carcinoma treated at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital throughout the years 1989-1998. The clinical diagnosis of ovarian carcinoma in the Finnish patients was made at the mean age of 56 years ± 14. The analysis of the Finnish control material was reported earlier (Enattah et al. 2002).

Polish samples consisted of 303 patients with ovarian carcinoma treated at the Department of Chemotherapy, Regional oncology Centre, Szczecin, during the years 2001-2004. The mean age of diagnosis was 54 (range 21-80) years. Control material consisted of 296 unselected adults.

The 152 Swedish samples were obtained from the southern Swedish health care region and were diagnosed with ovarian carcinoma during 1998-2000. The mean age of diagnosis was 59 (range 22-82) years. This patient material has previously been examined for germline mutations in *BRCA1* and *BRCA2*. The control samples were obtained from healthy individuals and from patients without known malignancy operated on at the Department of Orthopedics at the University Hospital in Lund.

4.1.5 Ethical considerations

The Ethics Committee of Helsinki University Hospital approved the lactase studies with a decision numbered 523/E7/2001. Written informed consent was obtained from the patients and controls. The ovarian carcinoma project has been approved by the Ethics Committee of the Department of Obstetrics and Gynecology, HUCH (040/95) and National Authority for Medicolegal Affairs (1129/32/300/03). As regards fresh tissue and blood samples informed consent has been obtained from each patient. The study was done in accordance with the Helsinki declaration.

Methods used in this study have been described in the original articles below and listed in Table 3.

Table 3. Methods used in the study.

Methods	Original publication
Bioinformatics used	I
DNA extraction with a strip method	III
RNA extraction	III
Polymerase chain reaction	III
Genotyping by Solid-phase minisequencing	IV
Quantitation by Solid-phase minisequencing	III
RT-PCR	III
Electrophoresis:	
Agarose gel electrophoresis	III
ABI377	III

4.1.6 Methods not described in the publications

4.1.6.1 Sequencing

The PCR products (~5 µl) were cycle sequenced in a total volume of 10 µl on both strands with 4 µl BigDye™ Terminator RR mix, (Applied Biosystems, Perkin Elmer, Foster City, CA, USA) and 3.5 pmol of primers. The sequencing reaction included a 30-second denaturation step at +96°C, a 15-second primer-annealing step at +55°C and a 4-minute extension step at +60°C. The sequencing reactions were cycled 25 times and stored at +4°C. Sequenced products were electrophoresed on an ABI377 or ABI3730 DNA Sequencher (Perkin Elmer).

4.1.6.2 Microsatellite genotyping

One of the two PCR primers of each reaction included a fluorescent label (6-FAM, TET, HEX) at its 5' end. PCR products were pooled and electrophoresed with TAMRA 500 size marker (Applera Corporation, Norwalk, CT, USA) on an ABI 377 (Applied Biosystems) using a 6% polyacrylamide gel. The GENESCAN 3.0 program together with GENOTYPER 2.0 (Applera Corporation) was used for determining the genotypes. The genotypes were validated by two individuals, and organized using LINKBASE 0.952 (Suomalainen 1998 unpublished). The pedigree inconsistencies were checked by PEDCHECK 1.1 (O'Connell and Weeks 1998).

4.1.6.3 Statistical analyses

Parametric two-point linkage analyses were performed with the recessive mode of inheritance assuming complete penetrance and 0.001 disease allele frequency. The analyses were carried out with the MLINK program of the linkage package (Lathrop and Lalouel 1984; Lathrop et al. 1984) using the ANALYZE 2.1 program (Hiekkalinna et al. 2005). Association analyses were carried out using the haplotype relative risk likelihood-ratio algorithm based test (HRR-LRT). In addition, LD was measured by a likelihood based lambda (λ) test which gives information about the proportion excess of a certain allele in disease chromosomes, relative to its population frequency (Terwilliger 1995). Haplotypes were constructed using the GENEHUNTER 2.1 program (Kruglyak et al. 1996) and the CLD founder haplotype was generated assuming that there were a minimum number of recombinations.

5 RESULTS AND DISCUSSION

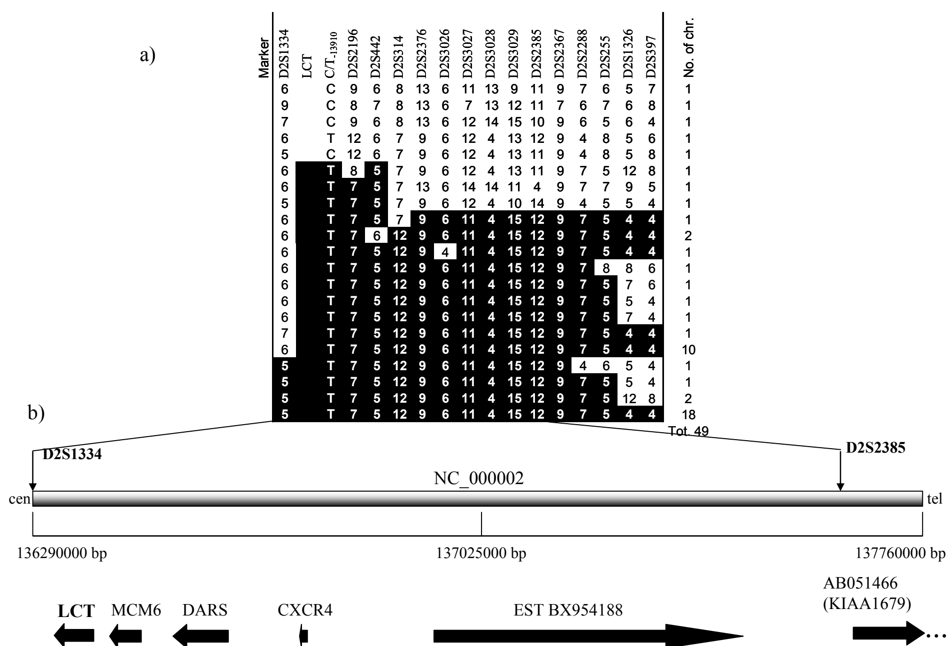
5.1 Results of congenital lactase deficiency (I)

Previously, the CLD locus was assigned by linkage analysis on 2q21. LD and haplotype analysis appeared to restrict the critical locus between microsatellites D2S314 and D2S2385 (Järvelä et al. 1998). Hence, the locus excluded *LCT* as a causative gene. The observation was supported by the sequence analysis of the coding region of *LCT* that not succeeded in finding disease mutations in a Finnish CLD patient (Poggi and Sebastio 1991). However, sequence analyses of transcripts between D2S314 and D2S2385 could not identify disease-causing mutations (data not shown). In this study, the CLD locus was further analysed using a denser microsatellite map. In addition, five novel CLD families were included in the analyses.

5.1.1 Linkage, linkage disequilibrium and haplotype analyses

Fifteen microsatellite markers and C/T_{-13910} were genotyped covering 5.88 cM on 2q21-2q22 in 24 families with a total of 32 affected children. Twenty-one different haplotypes were detected in 48 disease chromosomes (Figure 12 a). The highest parametric two-point LOD score of 7.24, with a θ value of 0, was achieved with marker D2S2385. Strong evidence for linkage with LOD scores $\geq$2.95 was obtained with all the included markers. LD was evaluated by calculation of the allelic association using HRR-LRT and lambda (λ) methods. The HRR-method gave statistically the most significant p-values of 1×10^{-10} and 9×10^{-10} with markers D2S314 and D2S2385, respectively. A significant LD was observed with all markers except D2S1334 and D2S2288, of which p-values were higher than 0.01. In contrast, a λ-value 0.84 gave the strongest evidence for LD with marker D2S3026 between the previous markers, decreasing to the lowest value of 0.30 at marker

D2S1334. The founder haplotype, cen-5-T_{-13910}-7-5-12-9-6-11-4-15-12-9-7-5-4-4-tel, was present in 18 disease chromosomes from which 15 of the remaining 20 different haplotypes have been derived (Figure 12 b). Five disease chromosomes each of which had a diverged haplotype and could not linked to the major haplotype. Haplotypes was detected to share a region that includes *LCT* and we carried on to perform sequence analysis of this gene. The haplotype and transcript map of the critical DNA region for CLD is shown in Figure 12.



Marker D2S1334	LCT	C/T_{-13910}	D2S2196	D2S442	D2S314	D2S2376	D2S3026	D2S3027	D2S3028	D2S3029	D2S2385	D2S2367	D2S2288	D2S255	D2S1326	D2S397	No. of chr.
6		C	9	6	8	13	6	11	13	9	11	9	7	6	5	7	1
9		C	8	7	8	13	6	7	13	12	11	7	6	7	6	8	1
7		C	9	6	8	13	6	12	14	15	10	9	6	5	6	4	1
6		T	12	6	7	9	6	12	4	13	12	9	4	8	5	6	1
5		C	12	6	7	9	6	12	4	13	11	9	4	8	5	8	1
6		T	8	5	7	9	6	12	4	13	11	9	7	5	12	8	1
6		T	7	5	7	13	6	14	14	11	4	9	7	7	9	5	1
5		T	7	5	7	9	6	12	4	10	14	9	4	5	5	4	1
6		T	7	5	7	9	6	11	4	15	12	9	7	5	4	4	1
6		T	7	6	12	9	6	11	4	15	12	9	7	5	4	4	2
6		T	7	5	12	9	4	11	4	15	12	9	7	5	4	4	1
6		T	7	5	12	9	6	11	4	15	12	9	7	8	8	6	1
6		T	7	5	12	9	6	11	4	15	12	9	7	5	7	6	1
6		T	7	5	12	9	6	11	4	15	12	9	7	5	5	4	1
6		T	7	5	12	9	6	11	4	15	12	9	7	5	7	4	1
7		T	7	5	12	9	6	11	4	15	12	9	7	5	4	4	1
6		T	7	5	12	9	6	11	4	15	12	9	7	5	4	4	10
5		T	7	5	12	9	6	11	4	15	12	9	4	6	5	4	1
5		T	7	5	12	9	6	11	4	15	12	9	7	5	5	4	1
5		T	7	5	12	9	6	11	4	15	12	9	7	5	12	8	2
5		T	7	5	12	9	6	11	4	15	12	9	7	5	4	4	18
																	Tot. 49

Figure 12. a) Haplotypes of 24 Finnish CLD families on 2q21. Haplotypes were constructed using 15 microsatellites and C/T_{-13910}. Haplotypes derived from one major haplotypes are highlighted.

b) Transcript map of the critical DNA region for CLD. Lactase (*LCT*), minichromosome maintenance deficient 6 (*MCM6*), aspartyl-tRNA synthetase (*DARS*), chemokine receptor type 4 (*CXCR4*) and KIAA1679 are the known genes in the region (indicated by horizontal arrows). The centromere is on the left.

5.1.2 CLD mutations in *LCT*

The *LCT* gene was sequenced in all 32 CLD patients in 24 families and a total of five different mutations were identified. Twenty-seven patients out of thirty-two were homozygous for a substitution of T→A at c.4170 that was designated as the $\text{Fin}_{\text{major}}$ mutation (Table 4, Figure 13). All their parents analysed (five were not available) and five patients were c.4170T→A heterozygotes. The c.4170T→A mutation was predicted to result in a premature stop codon Y1390X in exon nine, thus leading to a truncation of the eight remaining *LCT* exons. Two patients out of those five $\text{Fin}_{\text{major}}$ heterozygotes had a paternally transmitted deletion of four nucleotides, c.4998_5001delTGAG, in their other disease allele in exon 14. This mutation was anticipated to end in a frameshift and an early stop codon after 55 changed amino acids (S1666fsX1722). One of those five patients had a deletion of two nucleotides c.653_654delCT in exon two predicting a frameshift and truncation at S218fsX224. The patient's father carried the mutation. The last two mutations, each of which was found in a different patient, were substitutions of c.804G→C and c.4087G→A. They are expected to result in amino acid substitutions of histidine for glutamine at Q268H and serine for glycine at G1363S in exon 3 and 14, respectively. Two patients were heterozygous for Q268H or G1363S and $\text{Fin}_{\text{major}}$. Both mutations were detected to be as a maternal origin. In addition, the c.4087G→A mutation was carried by a healthy sibling in the family. The CLD mutations and corresponding lactase activities of duodenal biopsy specimens are shown in Table 4.

Table 4. Identified CLD mutations in the *LCT* gene and detected lactase activities of duodenal biopsy specimens.

Mutation	Consequence in protein	Exon	Mean lactase activities U/g protein (range)
c.4170T→A	Y1390X	9	2.4 (0-7)
c.4998_5001delTGAG	S1666fsX1722	14	5 (3-7)
c.653_654delCT	S218fsX224	2	2
c.804G→C	Q268H	3	5
c.4087G→A	G1363S	9	3

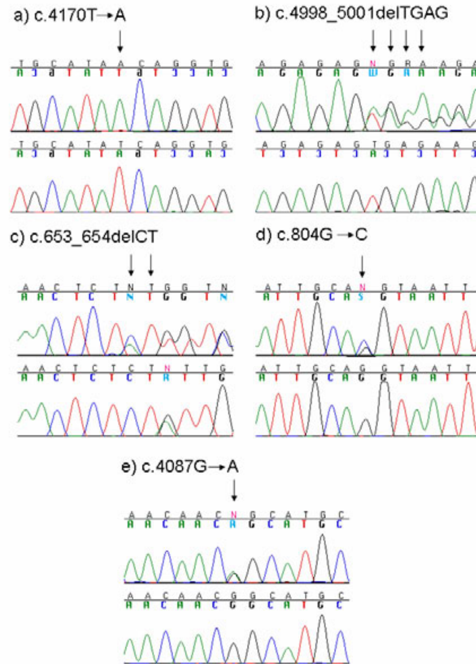


Figure 13. CLD mutations in the *LCT* gene. DNA sequence chromatograms of five identified CLD mutations, a-e. Mutation Y1390X (a) is homozygote, whereas the rest of mutations are heterozygotes. The first row shows affected and the second row wild type sequences.

5.1.2.1 The impact of Fin_{major} on *LCT* transcription

To explore the consequence of the Fin_{major} mutation at the mRNA level, *LCT* transcripts were analyzed using total RNA extracted from duodenal biopsies of a five-year old patient and five control subjects aged 6.7-17 years (Table 1, Study II). The patient was heterozygous for Fin_{major} and C/T_{-13910} that defines the lactase non-persistence/persistence phenotype. The patient's duodenal lactase activity was 6 U/g of protein and the L/S ratio was 0.08. All control subjects had the C/T_{-13910} genotype and lactase activity (determined from four subjects) ranged between 21-29 U/g of protein and L/S ratio between 0.28-0.62. RT-PCR followed by solid-phase minisequencing was employed to quantitate mRNA levels using an informative SNP G/A_{+593} in *LCT*. The T_{-13910} allele of the control subjects was expressed about five times higher than the downregulated C_{-13910} allele. On the contrary, the T_{-13910} allele carrying the Fin_{major} was not able to maintain the *LCT* expression. The phenomenon is probably due to nonsense-mediated mRNA decay (NMD). This mechanism is harnessed to eliminate the production of potentially harmful truncated proteins degrading the nascent mRNA (Maquat 2005).

5.1.3 Genealogy and carrier frequencies of the CLD mutations in Finland

On the basis of the genealogical studies Fin_{major} and S1666fsX1722 mutations were tracked to central and northern Finland, respectively. Mutations S218fsX224, Q268H and G1363S were traced in eastern Finland. The carrier frequency for the CLD mutations was determined among 556 anonymous donors representing both the early and the late settlement regions of Finland (Figure 14). Carriers of the Fin_{major} were found in all towns analyzed locating in later settlement regions. The highest carrier frequency of was found in Nilsiä 1:35 (4/140) predicting an incidence of ~1:4800 for CLD in this particular region. In this region, a cluster of the ancestors

was also trailed using church book records (Järvelä et al. 1998). No carriers for the four more rare CLD mutations were observed in any subpopulation screened.

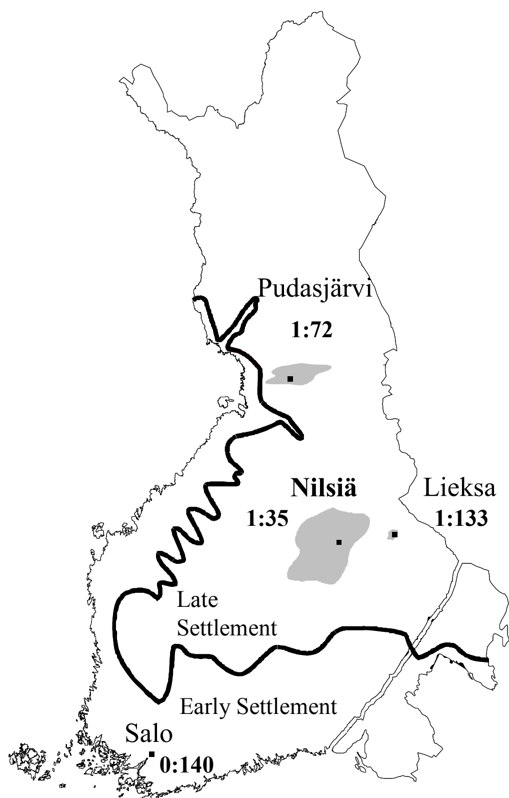


Figure 14. Carrier frequency of the Y1390X mutation in four subpopulations of Finland. Modified from Study I.

5.2 Discussion of congenital lactase deficiency

CLD patients suffer from deficiency of lactase activity in the intestinal lumen. Thus, infants, fed breast or other lactose containing milks, are exposed to substantial watery diarrhea that risks normal growth and development. Breast milk is, indisputably, an essential source of nutrition postnatally. For example, antibodies of

mother's milk stimulate infants' immune systems to protect them from fatal infections. The importance of lactose is indicated by the fact that breast milk contains 6.5% of lactose that represents 37% of its energy resource (Fineli, National Public Health Institute, Finland). Furthermore, acidic lactose facilitates the development of the valuable colonic bacterial floor of newborns.

It took nearly a half of the century to unravel the molecular background of CLD as the very first patients were described already in 1959 (Holzel et al. 1959). In this study, five distinctive mutations in the *LCT* gene underlying this disease are finally reported. The mutation Y1390X was found to be the prevailing one as 90% (43/48) of disease chromosomes carried the mutation. The observation is considered to reflect the founder effect of the Finnish population. The rest of the mutations were rare being specific to one or two families only.

As described in chapter 2.2.3 lactase is synthesized as a precursor of 1927 amino acids. The lactase precursor includes a pro-domain (repeats I and II) and an active domain (repeats III and IV), which possesses the catalytically active sites for phlorizin hydrolase and lactase (Figure 15). The pro-domain functions as an intermolecular chaperone assisting in the formation of the functional enzyme complex. It is cleaved off in the Golgi complex yielding the mature enzyme. Mutations G1363S, Y1390X (Fin_{major}) and S1666fsX1722 hit directly in the active domain while S218fsX224 and Q268H were found in the pro-domain. Fin_{major} and S1666fsX1722 truncate lactase polypeptide at repeat IV and S218fsX224 at repeat I. Fin_{major} was observed not be able to retain *LCT* expression probably affecting the stability of *LCT* mRNA directing the mutated transcript to NMD. NMD is across eukaryotes a conserved mRNA surveillance mechanism that verifies that mRNA has the appropriate quality for translation. NMD prevents the production of potentially fatal truncated proteins created by premature termination codons (Wagner and Lykke-Andersen 2002; Maquat 2005). However, premature termination codons closer than 50 nucleotides upstream of the last intron escape from NMD. This allows

accumulation of mutant protein resulting in a dominant negative effect as observed in dominant forms of β-thalassemia and Marfan syndrome (Baserga and Benz 1988; Frischmeyer and Dietz 1999). Although, NMD has been observed to link with nonsense mutations they hardly had enough evolutionary pressure to maintain the mechanism. Indeed, NMD has been observed to regulate normal cellular processes and mute genomic noise (Carter et al. 1995; Mendell et al. 2004). Lactase activity and its mobility on polyacrylamide gels were studied in duodenal biopsies of three of our CLD patients with Fin_{major}. In this preliminary study, brush-border lactase protein and activity was barely detectable, which supports undisputedly the NMD hypothesis (Freiburghaus et al. 1976). Alleles with frameshift mutations, S218fsX224 and S1666fsX1722, resulting eventually in stop codons may also be guided to NMD. Q268H substitutes the alcaline histidine for an uncharged glutamine and was found in LCTα the intramolecular chaperone. Q268H may affect the structure of LCTα disturbing the maturation process of lactase polypeptide. G1363S substitutes a small glycine for a reactive serine. The mutation locates close to catalytically active sites possibly changing the three-dimensional structure of the LCT polypeptide hence, inactivating the enzyme. The importance of the Q268 and G1363 residues are indicated by the fact that they are conserved across the species. The schematic structure of lactase polypeptide and the location of the five CLD mutations are illustrated in Figure 15.

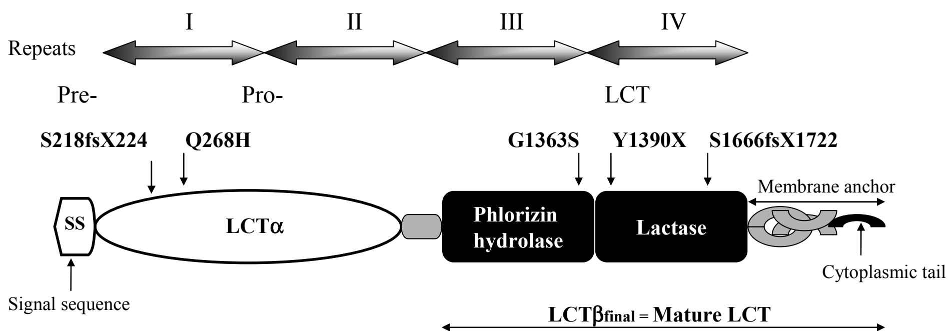


Figure 15. Structure of the translated lactase polypeptide and the location of the identified CLD mutations. The primary translation product (pre-pro-LCT) contains 1927 amino acids. The pro-LCT consists of four homologous repeats (I-IV). Repeats I and II are located in the pro-domain while repeats III-IV are found in the mature lactase (Mantei et al. 1988). Mutations S218fsX224 and Q268H were identified in LCTα that functions as an intramolecular chaperone. Mutations G1363S, Y1390X and S1666fsX1722 were found in mature lactase.

Regional clusters of CLD in the late settlement region of Finland, the rareness of disease, and the detected ~6 cM interval for LD indicate a fairly late introduction of the disease alleles into the population. The timing is expected to coincidence with many of the diseases in the Finnish disease heritage like IOSCA whose disease alleles were estimated to have been introduced before the major internal population movement to the inland in the 1500s some 30 generations, 750 years ago (Varilo et al. 1996).

Contrary to the rare CLD, lactase non-persistence is a common enzyme deficiency worldwide. Lactase activity in adult-type hypolactasia declines to a mean value of 5.9 U/g protein (Study III) differing significantly from the CLD patients' mean value of 2.4 U/g protein [$t(36) = 5.0$; $p = 0.0001$]. Interestingly, the CLD Fin_{major} mutation was found in the background of the lactase persistent allele, T_{-13910}, in Finns. As showed in this study, some Fin_{major} carriers with the C/T_{-13910} genotype may suffer from lactose-related symptoms after downregulation of the C_{-13910} allele.

5.3 Results of *LCT* mRNA studies

5.3.1 *LCT* expression and quantitation in children (II)

Fifteen subjects of forty-five (33%) were observed to be heterozygous for the A/G_{+593} polymorphism residing in exon 1 of the *LCT* gene and, thus their intestinal biopsies could be used for further analyses. Twelve of them were heterozygous for the lactase persistent C/T_{-13910} and three were homozygous for the lactase non-persistent C/C_{-13910} genotype (Table 5). The subjects with the C/T_{-13910} genotype (age range: 10 months to 17 years) had high lactase activity, ranging from 21 to 113 U/g of protein.

Table 5. Lactase activities, L/S and allelic ratios in children.

Age (years)	Genotype (C/T_{-13910})	C_{-13910} expressed (%)	Lactase activity (U/g protein)	L/S ratio
1.1	C/C	52	ND	ND
5.0	C/C	51	24	0.28
22.8	C/C	49	6	0.08
0.8	C/T	48	85	1.11
1.1	C/T	52	113	1.02
4.0	C/T	48	31	0.49
4.3	C/T	42	53	0.48
4.7	C/T	40	40	0.62
4.9	C/T	42	ND	ND
6.7	C/T	18	22	0.28
7.6	C/T	16	ND	ND
11.1	C/T	13	29	0.54
14.9	C/T	17	21	0.40
17.0	C/T	24	29	0.62

ND not determined

A standard curve was prepared for the G/A_{+593} of the *LCT* gene. An equal level of lactase mRNA was transcribed from both C_{-13910} and T_{-13910} allele in children younger than 5 years. In those aged between 4 and 5, a decline in the lactase mRNA transcribed from the C_{-13910} allele started to appear. In the children aged four to five years, the relative expression from the C-allele was 48% in two cases and, 40-42% in another three. In children aged six and seven years relative *LCT* mRNA expression was reduced to 18% and 16%. In children >6 years of age the relative expression was reduced to 13-24 % of the expression from that of the T_{-13910}-allele (see Figure 16, and Table 5). In contrast, in children with the C/C_{-13910} genotype the relative expression from both alleles was equal as expected.

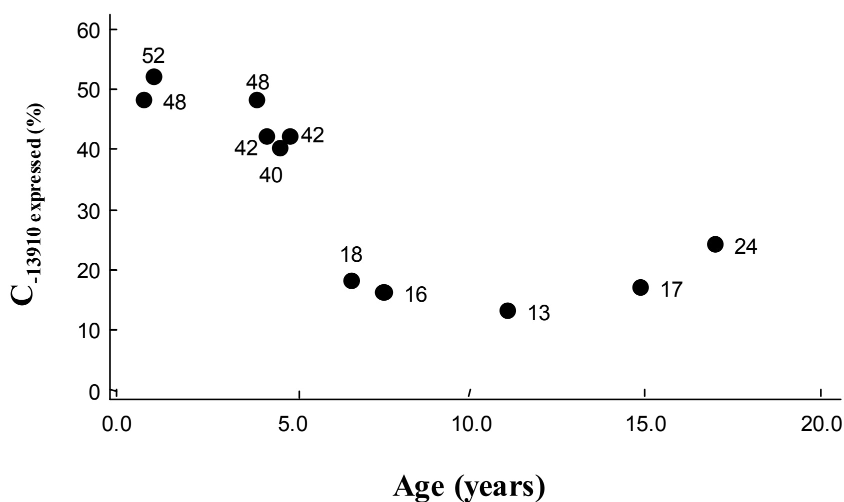


Figure 16. Relative proportion of *LCT* expressed by the C_{-13910} allele.

5.3.2 *LCT* expression in adults (III)

Relative allelic expression of *LCT* transcripts was studied using 52 patients. Eleven of the 52 patients (21%) were observed to be hypolactasic with lactase activities (4-9 U/g), L/S ratios (0.05-0.18), and genotypes C/C_{-13910} and G/G_{-22018}. Twenty-three (44%) patients' lactase activities ranged between 13-49 U/g, their L/S ratios from

0.35 to 0.69 and they were heterozygous for C/T$_{-13910}$ and G/A$_{-22018}$. Eighteen of the 52 (35%) patients were homozygous for T/T$_{-13910}$ and A/A$_{-22018}$ and their lactase activities ranged from 18 to 87 U/g and their L/S ratios from 0.49-1.05 (Figure 17). Using Fisher's exact test, the statistically significant difference between each genotype and their corresponding L/S ratios was found to have a p-value <0.0001.

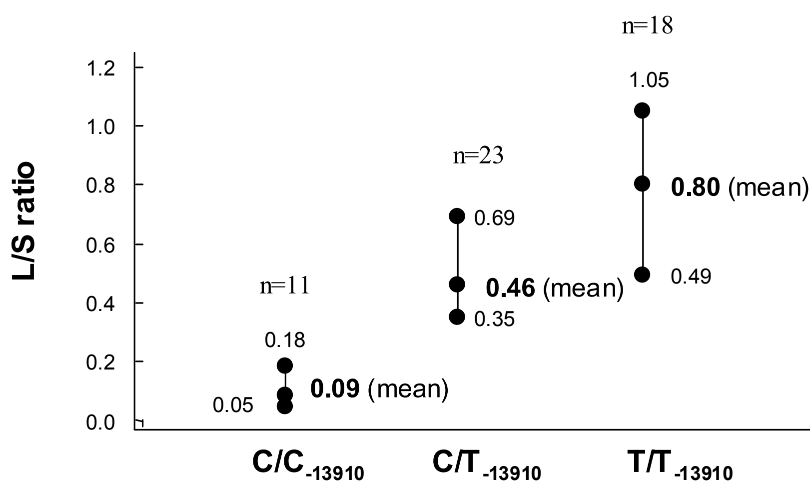


Figure 17. Correlation of *LCT* genotypes with L/S ratios. The effect of the T$_{-13910}$ variant was observed as elevation of L/S ratio, ($p<0.0001$).

5.3.2.1 Quantitation of *LCT* mRNA levels

The most informative single nucleotide polymorphisms (cSNPs) in the coding region of the *LCT* gene are located in exon 1 (c.593G→A) and exon 17 (c.5578C→T) in the Finnish families. These cSNPs were primarily chosen to quantify the relative mRNA levels. Twenty-two of fifty-two (42%) were found to be heterozygous for one or both of these cSNPs. Novel single nucleotide polymorphisms were searched for in the 30 uninformative patients by direct sequencing of exons 2, 6, 10, and/or 13. Six patients were found to be informative at least for one of the sites. Seventeen of the remaining 24 patients, were homozygous for T/T$_{-13910}$, A/A$_{-22018}$, 7 of those 24 were heterozygous for C/T$_{-13910}$, G/A$_{-22018}$.

Thus, 11 hypolactasic patients, 16 lactase persistent heterozygous and one lactase persistent homozygous could be further analysed for relative allelic expression.

To relate the actual difference in the expression of the *LCT* alleles in the minisequencing assay a standard curve was prepared for the G/A_{+593} cSNP in exon 1. The relative quantitation of the *LCT* alleles showed that the persistent allele represented 92% +/- 6% (mean +/- standard error of mean, range 78-99%, n=14) of the total *LCT* mRNA quantity. A clear increase in the R-value, suggesting allelic asymmetry of *LCT* mRNA expression, was also seen independently when quantitation was performed for any of the cSNPs located in exons 1, 2, 6, 10, 13, or 17 (Table 6). Hypolactasic patients with the C/C_{-13910} and G/G_{-22018} genotypes had equal expression of both alleles (47% +/-1%, n=7). Only one subject homozygous for lactase persistent T_{-13910}, A_{-22018} had an informative SNP of the coding region of the *LCT* gene. This individual equally expressed both *LCT* alleles (Table 6).

Table 6. The genotypes of each patient and their expressed allelic ratio of the *LCT* gene.

		Genotype	Cpm ratio of the cSNPs of the LCT gene				
Diagnosis	N	C/T_{-13910}/ G/A_{-22018}	Exon1 A/G	Exon6 G/T	Exon10 G/A	Exon13 C/T	Exon17 C/T
Hypolactasic	11/11	CC/GG	0.58-0.70	-	1.1	1.3-1.4	1.5-4.7
Lactase persistent	16/23	CT/GA	3.3-37	-	5.5-7.6	-	13-64
Lactase persistent	1/18	TT/AA	-	1.6	-	-	-

5.4 Discussion of *LCT* mRNA studies (II, III)

The decline of lactase activity after childhood, adult-type hypolactasia, is probably the best-known enzyme deficiency in humans. Hence, it has inspired scientists to study its occurrence for a century. The development and creation of methods in the

natural science over the last two decades has provided opportunities to approach this dilemma at the molecular level. As discussed in section 2.4.3.2 ‘Mechanism of downregulation’, the downregulation of lactase activity has been suggested to operate at the transcriptional and/or posttranscriptional level. This enigma climaxed in Enattah and his colleagues’ (2002) linkage disequilibrium and haplotype studies in Finnish families, which exposed two variants associated with adult-type hypolactasia supporting the transcriptional regulation theory.

In this study, those variants affecting the developmental changes in *LCT* expression was evaluated in biopsy specimens by determining the biological activities of lactase and sucrase and quantitating the mRNA levels by solid-phase minisequencing in Finnish children and adults. In children the C_{-13910} allele, determining lactase non-persistence, declined by the age of six years (Figure 18). In children aged six years or more the C_{-13910} allele contributed only 13-24% to the produced *LCT* mRNA. The age of *LCT* mRNA was observed to decline is pretty well to scale with that of the age of downregulation of lactase activity in Finnish children (Sahi 1994a; Rasinperä et al. 2004). However, the age when downregulation takes place varies between populations. In Thai children the downregulation event was observed between one and two years of age (Keusch et al. 1969a). In African children, the decline of lactase activity was detected from three years of age (Rasinperä et al. 2004). A British study of different ethnic groups demonstrated that the decline of *LCT* mRNA expression was detected from the second year of life (Wang et al. 1998b). The spatial and temporal gene regulation involved in the timing of *LCT* decline is so far unsolved. Timing variables should be taken into account when the usefulness of the genetic testing of C/T_{-13910} for adult-type hypolactasia is estimated. Tests before the age of downregulation occurs can lead to diminished milk consumption too early. Thus, positive effects of milk may be remained unexploited. The determination of C/T_{-13910} after 8-12 of years is considered to reflect the lactase phenotypes reliably (Rasinperä et al. 2004). In adults, the effect of a persistent allele T_{-13910} A_{-22018} was seen in elevation of lactase activity that was furthermore observed in significant

correlations in lactase activities between all the three genotypes C/C_{-13910} G/G_{-22018}, C/T_{-13910} G/A_{-22018}, and T/T_{-13910} A/A_{-22018}. The quantitation of relative *LCT* mRNA levels showed a clear difference between patients with lactase persistent and non-persistent genotypes. The lactase persistent allele, T_{-13910} A_{-22018}, had an average 11.5-fold higher expression than the non-persistent allele, C_{-13910} G_{-22018} (Figure 18). Lactase non-persistence patients having the C/C_{-13910} G/G_{-22018} genotype, and patients homozygous for lactase persistent with the T/T_{-13910} A/A_{-22018} genotype evenly expressed both alleles. These observations unambiguously show that the variants C/T_{-13910}, G/A_{-22018}, associated with adult-type hypolactasia, are also associated with the transcriptional regulation of the *LCT* gene. These results agree with previous findings that indicated that the decline of lactase activity is regulated at the transcriptional level (Escher et al. 1992; Lloyd et al. 1992; Fajardo et al. 1994) operating by a cis-acting element (Wang et al. 1995; Wang et al. 1998b).

Both the SNPs, C/T_{-13910} and G/A_{-22018}, are located in introns of *MCM6*, which locate at 5'-end of *LCT*. MCM6 is a mammalian homologue of mis5 of yeast, it functions as a cell cycle factor (Takahashi et al. 1994). *MCM6* is expressed in a variety of human tissues including intestine. Intestinal expressions of *MCM6* and *LCT* were not observed to correlate with subjects of lactase persistence or non-persistence suggesting that these genes are independently regulated (Harvey et al. 1996). So, obviously the influence of the *MCM6* gene on adult-type hypolactasia is only structural. The observation was confirmed later when the C/T_{-13910} construct was not able to activate the MCM6 promoter (Lewinsky et al. 2005).

Recently, studies have been focussed to clarify the functional role of the C/T_{-13910} G/A_{-22018} variants. Two studies using human (Troelsen et al. 2003b) and rat (Olds and Sibley 2003) LCT promoter-reporter gene construct analyses with C/T_{-13910} region in Caco-2 cells demonstrated independently differential regulation of LCT promoter activity. Furthermore, EMSA showed that the observed difference was a consequence of the binding capacity of the nuclear factors for the C_{-13910} and T_{-13910}

allele constructs (Troelsen et al. 2003b). The T_{-13910} variant had a stronger interaction than C_{-13910} suggesting a model for the T_{-13910} as a strong transcriptional enhancer. Similar analyses of the G/A_{-22018} variant did not reveal any significant functional role for phenotype differences of lactase persistence/non-persistence (Olds and Sibley 2003; Troelsen et al. 2003b). Later, DNA affinity purification analyses showed that Oct-1 binds directly to the C/T_{-13910} variant co-purifying with GAPDH and furthermore, the binding was detected to be stronger to the T_{-13910} variant than the C_{-13910} variant. DNase footprint and supershift analyses identified binding motifs for Cdx-2, GATA-6, Fox and HNF-4α in the close proximity of C/T_{-13910}. In mutation analyses, GATA-6, Fox and HNF-4α motifs were detected to be necessary for the pursuit of the C/T_{-13910} enhancer. Co-transfection analyses with C/T_{-13910} demonstrated that Oct-1 was able to activate LCT promoter only with HNF-1α. Interestingly, a HNF-1α site has not been found within the C/T_{-13910} enhancer but it probably is acting via a proximal promoter, which has a HNF-1α binding site. The results are convincing. The C/T_{-13910} variant seems to locate in the heart of an enhancer structure consisting of Oct-1, GATA, Fox and HNF-4α binding sites (Figure 18). Oct-1 with the C/T_{-13910} sequence directs specifically the LCT promoter activity and enhances reporter gene expression with the T_{-13910} variant significantly over the C_{-13910} variant. These discoveries offer a plausible model for the T_{-13910}-induced lactase persistence phenotype in humans (Lewinsky et al. 2005).

Although, we have learned a great deal about the molecular background of the lactase persistence phenotype the virtual cascade that leads to activation of the *LCT* gene is still obscure. Interestingly, Oct-1 has been reported to recruit co-factors for chromatin structure enhancing or silencing genes depending on the tissue type or promoter structure (Zabel et al. 2002; Remenyi et al. 2004). A similar mechanism was also considered as a potential one for the lactase persistence phenotype (Lewinsky et al. 2005). The T_{-13910} allele has been demonstrated to match the functional, phenotypic and epidemiological data of European and European derived

populations (reviewed in section 2.4.3 ‘Genetics’). It has been reported that statistically the T_{-13910} allele could not explain the lactase persistence incidence in some African populations (Mulcare et al. 2004). If carefully characterized phenotypes can confirm the results, there are still unidentified variants to be found, which probably will provide their own interesting characteristics to explain the lactase persistence mechanism.

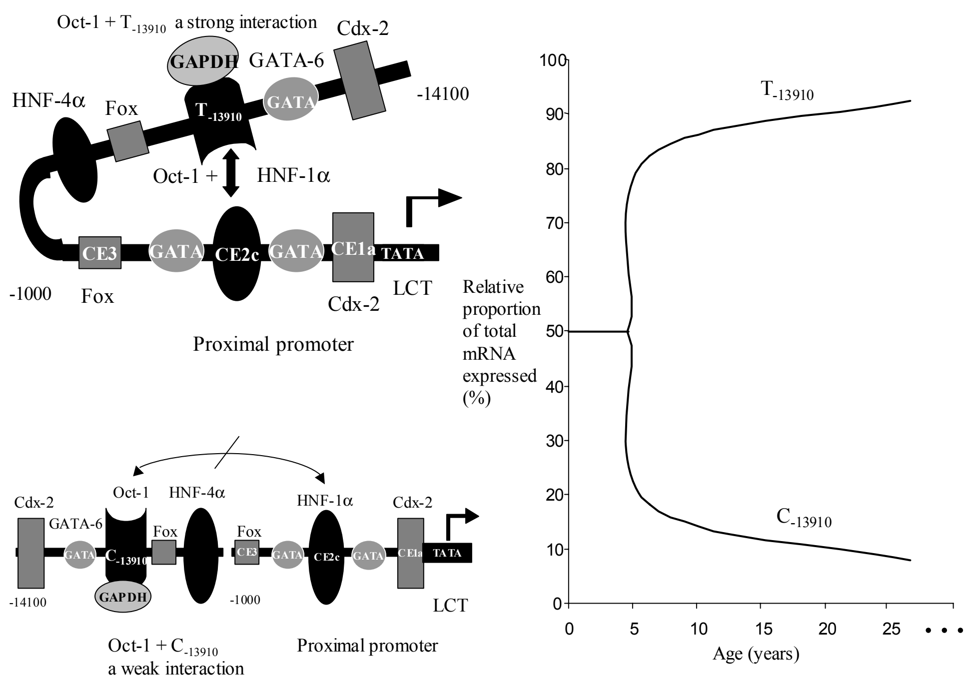


Figure 18. Regulation of the *LCT* gene – an overview. Regardless of the genotype of the C/T_{-13910} enhancer, *LCT* is fully expressed up to five years of age. The C_{-13910} variant has weaker interaction with Oct-1 than the T_{-13910} variant (Lewinsky et al. 2005), hence yet unknown mechanism allows the downregulation of the C_{-13910} allele at the age of six (Study II). In contrast, the T_{-13910} allele maintains *LCT* expression and is responsible for an average 92% of expressed *LCT* mRNA (Study III).

5.5 Results of lactase persistence and ovarian carcinoma (IV)

Lactase persistence had no statistically significant effect on the development of ovarian carcinoma in the Finnish, Polish or Swedish populations. Combined analysis of all three populations showed no significant risk for ovarian cancer (Figure 19). In addition, dissecting ovarian carcinoma tumours by histological subtypes for analyses did not affect the outcome (Table 1, Study IV).

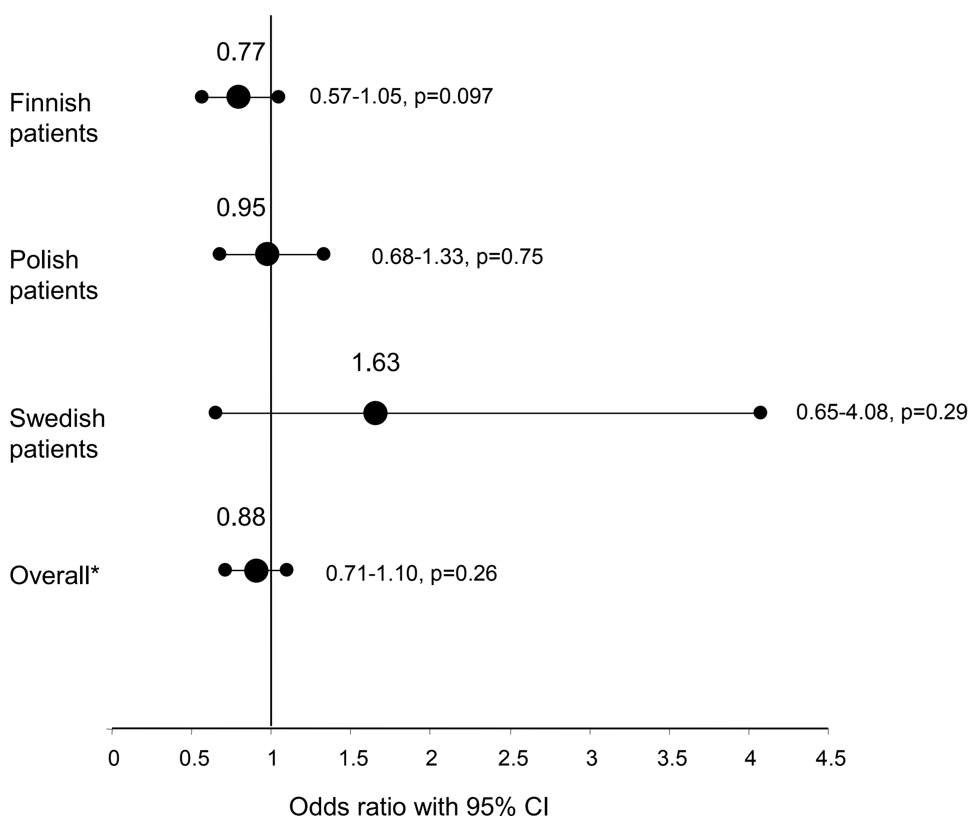


Figure 19. Molecularly defined lactase persistence and its risk to ovarian carcinoma pathogenesis in the Finnish, Polish and Swedish populations. *Mantel-Haenszel common odds ratio estimates, (CI) confidence interval.

The frequency of the C/C$_{-13910}$ genotype was detected as 33.1% in the Polish controls. This was a bit lower than the earlier reported incidence of lactase non-persistence (37.5%) diagnosed by BHT in the Polish population (Socha et al. 1984) In Swedish controls, the C/C$_{-13910}$ frequency was observed to be 10.3% which is in concordance with the recently observed occurrence of C/C$_{-13910}$ (9.6%) in the Swedish population (Nilsson TK personal communication).

5.6 Discussion of lactase persistence and ovarian carcinoma

Ovarian cancer is a deadly disease among women with a high incidence in western industrialized countries (World Cancer Research Fund 1997). Epithelial ovarian cancer (ovarian carcinoma) is the major cancer type explaining 90% of ovarian cancer cases. The aetiology is still for the most part unravelled but both genetic and environmental factors affect the pathogenesis. The pathogenesis has been studied intensively tracing frequent ovulation cycles and hormonal issues as contributory factors to the disease. Dietary factors particularly a high consumption of milk (lactose) and lactase persistence which is closely related its consumption have been put forward as risk factors for ovarian tumour genesis (Cramer 1989). Galactose, the hydrolysing product of lactose, was observed to be toxic to oocytes in animals studies (Chen et al. 1981; Schwartz and Storozuk 1988). Thus, galactose has been hypothesized to promote the development of ovarian carcinoma. In order to test the hypothesis, the C/T$_{-13910}$ variant that specifies lactase phenotypes, was defined in ovarian carcinoma patients and compared with healthy controls in the Finnish, Polish and Swedish populations. We observed that lactase persistence had a modest reduced effect (OR 0.77) on ovarian carcinoma risk but the trend was not statistically significant. Lactase persistence did not associate with the ovarian carcinoma risk either in the Polish or Swedish populations. The results might be related to the fact that Finnish milk consumption is the highest in the world (The

World Dairy Situation 2003). Lactase persistence facilitate milk's use as a nutrition which has been noted as an increasing milk consumption (Obermayer-Pietsch et al. 2004; Rasinperä et al. 2004; Rasinperä et al. 2006). However, these observations cannot replace the dairy consumption data of subjects of this study. As reviewed in chapter 2.5.1.1 'Dairy consumption and ovarian carcinoma risk' many studies have tried to shed light on the risks or benefits of milk and its components for ovarian tumour genesis. But there is not been any breakthroughs concerning the issue. Meta-analyses have shown that only large prospective cohort studies show a modest increased risk between high milk consumption and ovarian carcinoma (Genkinger et al. 2006; Larsson et al. 2006). Interestingly, the same trend was observed in a meta-analysis between milk consumption and colorectal cancer (Norat and Riboli 2003). Thus, large sample sizes probably play a role in these kinds of studies.

Milk is a versatile nutrient and an important source of calcium and many other traces and vitamins (Table 2). For example, dietary calcium has been observed to decrease bone fracture risk and to prevent osteoporosis in adulthood (Gueguen and Pointillart 2000; Prentice 2004). Milk has also been regarded as benefit for early farmers since the lactase persistence mutation arouse 5000-10000 years ago. The selection power of lactase persistence is the strongest seen in the human genome (Cavalli-Sforza 1973; Heston and Gottesman 1973; Flatz and Rotthauwe 1977; Aoki 1986; Flatz 1987; Bersaglieri et al. 2004). Perhaps, an abstemious consumption brings the best out of milk also today. The results of our study do not support the hypothesis that lactase persistence promotes the development of ovarian carcinoma.

6 CONCLUDING REMARKS AND FUTURE PROSPECTS

There are two different types of lactase deficiencies in humans; a rare recessively inherited CLD and a common so-called adult-type hypolactasia with milder features. CLD belongs to the Finnish disease heritage and is caused by mutations detected in the translated region of lactase, which is responsible for hydrolysing lactose in the intestine. In this study, five unique mutations were found in 32 patients in 24 families. They all were expected to inactivate mucosal lactase activities. Mutation Y1390X, Fin_{major}, is anticipated to direct *LCT* transcripts to NMD that is typically the consequence of premature stop codons. A detailed analysis of impact of other four mutations on the development of CLD should be conducted in the future. Especially, Q268H and G1363S mutations can bring new insights for the mechanism of lactase maturation and for crucial sites of activities of lactase and phlorizin hydrolase. From the clinical viewpoint, this study has created a direct DNA-based diagnosis and a carrier identification of CLD that has thus far been relied on clinical symptoms completed by lactase activity analysis from duodenal biopsy specimens.

The development of adult-type hypolactasia, lactase non-persistence, is dependent on a variant C/T_{-13910} locating 13.9 kb upstream from *LCT* (Enattah et al. 2002). In this study, the C_{-13910} allele was detected to allow the transcriptional downregulation of *LCT*. The timing of the downregulation was detected at the age of six. In adults, the T_{-13910} allele represented a mean 92% of the expressed *LCT* mRNA when compared with the C_{-13910} allele. The C/T_{-13910} variant locates in the middle of enhancer element consisting of Oct-1, GATA, Fox and HNF-4α binding sites. Oct-1 was demonstrated to bind directly to the C/T_{-13910} site. It has been speculated that Oct-1 may induce chromatin changes and activate *LCT* (Lewinsky et al. 2005). The major goal of future functional studies will be to clarify which genes are directly involved in transcriptional downregulation of *LCT*. For example, evidence of

activated or inactivated genes can be traced from duodenal biopsies by an Affymetrix array. It remains also unknown which variants and/or mechanisms are at the background of lactase persistence in some African and Arabia nomads.

Milk is a significant part of the Northern European and American diet. Milk's lactose has been suggested to be a risk factor for the development of ovarian carcinomas. As lactase persistence is associated with increased milk consumption, C/T_{-13910} was tested in ovarian carcinoma patients and in the Finnish, Polish and Swedish populations. Lactase persistence did not associate with ovarian carcinoma risk in any of the populations tested. Multiple studies have been conducted in an attempt to shed light on dietary milk and its components in relation to ovarian tumour genesis (section 2.5.1.1). However, the results have remained controversial possibly reflecting the fact that milk has no cancer promoting effect on the ovary. On the other hand, components of milk are hard to dissect in this type of studies. Cell line studies are a more exact approach to study promoting or inhibiting factors of tumour growth. For example, high concentrations of 1,25 dihydroxyvitamin D has been detected to inhibit human ovarian cancer cell lines (Ahonen et al. 2000; Miettinen et al. 2004). However, only two case-control studies have shown that dietary vitamin D diminish ovarian carcinoma risk (Bidoli et al. 2001; Salazar-Martinez et al. 2002). Ovarian cancer is a deadly disease, about 110000 women per year perishes from the disease in the world (Parkin et al. 2001). Even if some genes have found to be causal for the disease, the background of the most of cases has remained obscure. It is probable that eventually efforts will recompense those involved in the research and their findings pave the way for risk factors and mechanisms underlying ovarian carcinoma. It is hoped that new diagnostic markers would permit more accurate and specific diagnosis and offer new tools for drug development for this lethal cancer.

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Mikko Kuokkanen

8 ELECTRONIC DATABASE INFORMATION

Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/

Baylor College of Medicine, http://searchlauncher.bcm.tmc.edu/

Expressed Sequence Tags Database, http://www.ncbi.nlm.nih.gov/dbEST/

Finnish Disease Database, http://www.findis.org./

Fineli, Finnish Food Composition Database, http://www.fineli.fi/index.php?lang=en

GenBank, http://www.ncbi.nlm.nih.gov/

GENSCAN, http://genome.dkfz-heidelberg.de/cgi-bin/GENSCAN/genscan.cgi

Genome Database, http://gdbwww.gdb.org/

Marshfield Medical Genetic Foundation, http://research.marshfieldclinic.org/

National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/

Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim/

Reference Sequence Database, http://www.ncbi.nlm.nih.gov/RefSeq/

SWISS-PROT, http://www.ebi.ac.uk/swissprot/

University of California, Santa Cruz, http://genome.ucsc.edu/

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